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1) **Transforming growth factor-beta: A general view**
AUTHOR: Lawrence David A
AUTHOR ADDRESS: Growth Factors Group, UMR 146 du CNRS, Inst. Curie, Batiment 110, Centre Universitaire, 91405 Orsay, France**France
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2) **Gene therapy by** TGF **-beta-** receptor **-IgG** Fc chimera **inhibited extracellular matrix accumulation in experimental glomerulonephritis**
AUTHOR: Isaka Yoshitaka (Reprint); Akagi Yoshitaka; Kaneda Yasumi; Yamauchi Atushi; Orita Yoshimasa; Ueda Naohiko; Imai Enyu
AUTHOR ADDRESS: Osaka Univ., Osaka, Japan**Japan
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MINH TAM DAVIS
ART UNIT 1642, ROOM 3A24, MB 3C18
272-0830

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THE ADJUVANT ACTIVITY OF NONIONIC BLOCK POLYMER SURFACTANTS

I. The Role of Hydrophile-Lipophile Balance

ROBERT HUNTER,[1] FAITH STRICKLAND, AND FERENC KÉZDY

From the Department of Pathology, Emory University, Atlanta, GA 30322, and the Department of Biochemistry, The University of Chicago, Chicago, IL 60637

We developed an experimental model for studying the physicochemical basis of the adjuvant activity of surface-active agents. Pluronic polyols are chemically similar but physicochemically diverse surface-active agents composed of polymers of hydrophilic polyoxyethylene and lipophilic polyoxypropylene. One of them, L121, was found to be a powerful adjuvant for increasing antibody formation to BSA in mice when injected in an oil-in-water emulsion. Another, L101, was less effective in increasing antibody formation, but was more effective in inducing granulomatous inflammation. Three others had little effect on either response. The ability of these and other surface active agents to serve as adjuvants correlated with a physicochemical parameter, the hydrophile-lipophile balance (HLB). All of the surfactants with strong adjuvant activity have HLB values of less than 2. They are strongly lipophilic and promote the retention of macromolecules by oil drops in oil-in-water emulsions. We propose that the adjuvant activity of these compounds is dependent on their ability to concentrate the adjuvant, immunogen, and host proteins on hydrophobic surfaces, where they are more effectively presented to cells of the immune system.

During the past several years, a great deal of progress has been made in defining the components of adjuvants that account for their activity. It has become increasingly clear that there exists a group of adjuvants that are surface-active agents. These are substances, such as detergents, that lower the surface tension of solvents or the tension between 2 immiscible phases such as oil and water. They have discrete hydrophilic and hydrophobic portions, and preferentially localize on hydrophobic surfaces in contact with aqueous media. The lipid A portion of endotoxin, lipoteichoic acid of Gram-positive organisms, and trehalose dimycolate (TDM)[2] of mycobacteria are surface-active agents and rank among the most potent of natural adjuvants (1, 2). Several hydrophobic cationic surfactants are strong adjuvants (3). Even lysolecithin has adjuvant activity (4). There is evidence that the adjuvant activity of these materials depends upon their surface activity. Analogs with smaller lipophilic groups, chemical modifications that reduce the polarity of hydrophilic groups, or a separation of the hydrophile and lipophile components have invariably reduced or abolished adjuvant activity (3, 5–7). However, many surface-active agents are not adjuvants. Consequently, few investigators have considered surface activity itself to be an important determinant of adjuvant action.

Many investigations have sought to identify active sites on adjuvant molecules. Since chemical modifications that change the surface activity of a molecule are likely to change its active sites and vice versa, the design of experiments to distinguish the roles of these factors is a nontrivial task. One approach is to identify the active site of an adjuvant, purify it, and synthesize analogues that contain the active site and have varying degrees of surface activity. This has been done with muramyl dipeptide (MDP), a water soluble, adjuvant active component of tubercle bacilli. If MDP is conjugated

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[2] Abbreviations used in this paper: HLB, hydrophile-lipophile balance; TDM, trehalose dimycolate; POP, polyoxypropylene; POE, polyoxyethylene; DTH, delayed-type hypersensitivity; LBSA, lipid-conjugated bovine serum albumin; L121, a Pluronic polyol surfactant; L101, a Pluronic polyol surfactant; L31, a Pluronic polyol surfactant; P103, a Pluronic polyol surfactant; F108, a Pluronic polyol surfactant; DDA, dimethyldioctadecylammonium bromide; OCT, octadecylamine; PBS, phosphate-buffered saline with 0.2% Tween 80; MDP, muramyl dipeptide.

with fatty acids, its adjuvant activity is increased (8). The effect can be produced with stearic acid, but the large complex mycolic acids are more effective. Consequently, one cannot be certain whether the increased adjuvant activity of mycolic acid-conjugated MDP is due to increased hydrophobicity of the long side chains or to additional active sites on them.

This paper reports a different approach. We sought chemically defined, synthetic materials with a wide range of quantifiable surface-active properties, but no differences in chemical constituents. The Pluronic polyols are simple block copolymers of polyoxypropylene (POP), which is hydrophobic, and polyoxyethylene (POE), which is hydrophilic (9). They are single, unbranched chains of a central polymer of POP flanked by 2 polymers of POE. By varying the overall size of the molecules and the ratios of POE to POP, a series of compounds have been produced that span a broad range of surface-active properties. They are considered to be among the least toxic of surface-active agents and are widely used in the food, drug, and cosmetic industries. Since they are simple polymers, it is difficult to hypothesize that differences in biologic activities among the Pluronic polyols could be due to the presence of active sites on 1 molecule that are not present on all of them.

Numerous methods have been developed for quantitating the properties of surface-active agents. The hydrophile-lipophile balance (HBL) is a particularly useful measure of the physicochemical properties of nonionic surfactants (Table I) (10). Agents with the highest HLB values are soluble in water and are used to solubilize lipids. Those with the lowest values, spreading agents, are soluble in oil and are used to spread thin films of oil on water. Agents with intermediate values have distinct functions as emulsifing or wetting agents. We hypothesized that HLB values would predict the adjuvant activity of both natural and synthetic surface-active agents in spite of their disparate chemical structures.

This hypothesis was tested by measuring the immune response to bovine serum albumin (BSA) injected with selected Pluronic polyols in oil-in-water emulsions. This type of emulsion was used because a natural surface-active adjuvant, TDM, exerts its greatest activity when prepared in such emulsions (11). Furthermore, surface-active agents exert their greatest physiocochemical activity when concentrated on surfaces, and finely dispersed emulsions maximize exposed surface area. Several Pluronic polyols and other surfactants were used to prepare emulsions with a light mineral oil, Drakeol 6VR, or with a paraffin wax, eicosane. Two proteins, BSA and lipid-conjugated BSA (LBSA), which selectively stimulates delayed-type hypersensitivity (DTH) in some animals (12), were also used. Several combinations of these materials were used in experiments to compare the stability of emulsions *in vitro* with their ability to form depots of antigen in tissue. Their immunogenicity for antibody and the intensity of the inflammatory responses at the site of injection and in the draining lymph nodes were assessed. A correlation was found between the HLB of surfactants and their adjuvant activity. These data form the basis of a new hypothesis about the mechanism of action of this type of adjuvant.

MATERIALS AND METHODS

BSA (Sigma Chemical Co., St. Louis, MO) was conjugated with dodecanoic acid to produce LBSA as described previously (12). Fifty of the 64 lysine amino groups on each LBSA molecule were conjugated with fatty acid. Both the BSA and LBSA were then labeled with ^{125}I by a standardized chloramine-T method to a specific activity of 0.5 mc/mg (13).

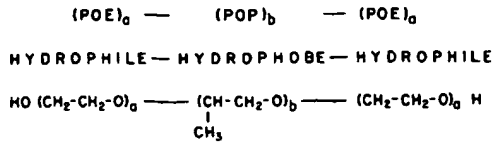
Surface-active agents. Five Pluronic polyols (L121, L101, P103, F108, and L31) were obtained from Dr. Irving R. Schmolka (BASF Wyandotte Co., Wyandotte, MI). The structure of these compounds is shown in Figure 1.

TABLE I

*HLB ranges and functional applications of nonionic surfactants**

HLB Range	Application
0–2	Spreading agent
3–6	Water-in-oil emulsifier
7–9	Wetting agent
8–15	Oil-in-water emulsifier
13–15	Detergent
15–18	Solubilizer

* Adapted from P. Becher (10) and I. R. Schmolka (personal communication).



Agent	MW POP	% POE
L 31	950	10%
L101	3250	10%
L121	4000	10%
P103	3250	30%
F108	3250	80%

Figure 1. Structure of Pluronic polyols. The central hydrophobic polymer of POP is flanked by two hydrophilic polymers of POE. Surfactants with a wide range of physicochemical properties are produced by varying the amounts and proportions of POP and POE. L stands for liquid, P for paste, and F for flakable solid, which are the physical forms of the pure surfactants at room temperature. The average molecular weight of the POP hydrophobic portion and the percent of the total molecular weight composed of POE are shown for each of the compounds used in these studies.

They are called block polymers rather than copolymers because the POP and POE portions occur in separate blocks. The particular compounds used in these studies were chosen because L101, P103, and F108 have identical hydrophobic portions and differ only in the length of the POE chains. L101, L121, and L31 have identical ratios of POP to POE and differ only in total m.w. Cationic surfactants with known adjuvant activity, dimethyldioctadecylammonium bromide (DDA) and octadecylamime (OCT), were obtained from Eastman Kodak Co., Rochester, NY.

Preparation and analysis of emulsions. Oil-in-water emulsions containing ^{125}I-labeled protein were prepared by a method similar to that developed by Ribi *et al.* (11) for emulsions of TDM using either Drakeol 6VR (Butler Refining Co., Butler, PA) or eicosane (Supelco Chemical Co., Bellefonte, PA). Drakeol 6VR is a light mineral oil composed of saturated hydrocarbons with an average chain length of 16 carbons. Eicosane is a saturated straight chain hydrocarbon 20 carbons long. Physically, it is a paraffin wax with melting point of 39°C. One hundred microliters of 1 of the hydrocarbons were placed in a teflon tissue homogenizer with 50 μl of surfactant and 1 mg of either lyophilized ^{125}I-LBSA or ^{125}I-BSA. The mixture was homogenized to form an oily paste that was emulsified in 2 ml of 0.2% Tween 80 in phosphate-buffered saline, pH 7.2 (PBS). Emulsions containing eicosane were prepared at 50°C. In order to evaluate their composition and stability, emulsions were placed in plastic vials, and the radioactivity was counted using a Beckman Biogamma counter. The oil and water phases were separated by centrifugation at 4°C and 3000 × G for 20 min. A sample of the PBS phase was removed using a 30-gauge needle and was counted. The proportions of antigen in the PBS and oil phases were calculated. Most of the remaining PBS was removed and the oil phase was resuspended in fresh PBS. This procedure was repeated at intervals to determine the rate of antigen release from the oil into the PBS. In other experiments, BSA was mixed with alum to yield 50 μg of BSA per 50-μl dose (14).

Immunization. Random bred ICR white mice, 8 to 10 wk old, were obtained from Laboratory Supply, Indianapolis, IN. They were immunized in the rear footpads with 50 μl of an adjuvant emulsion containing 25 μg of ^{125}I-labeled protein, 5 mg of hydrocarbon, and 2.5 mg of surfactant. They were bled at intervals through the retro-orbital plexus for antibody determinations. The popliteal lymph nodes and feet of some animals were fixed in 10% buffered formalin, counted in a gamma scintillation counter, sectioned, and stained with hematoxylin and eosin.

Antibody determinations. Hemagglutinin titers were measured using a 0.2% suspension of sheep red blood cells coated with BSA using gluteraldehyde (15). Antibody levels were also determined with a Farr type radioimmunoassay using 50% saturated ammonium sulphate to precipitate complexes of ^{125}I-labeled BSA and anti-BSA (16).

RESULTS

Retention of antigen in vitro and in vivo. Oil-in-water emulsions were prepared with equal amounts of ^{125}I-labeled BSA, a Pluronic polyol, and either Drakeol or eicosane. The proportions of the BSA label retained by the oil drops immediately and 7 days after preparation are shown in Table II. The large hydrophobic polyols with low HLB values, L121 and L101, promoted the retention of ^{125}I-labeled BSA or LBSA by drops of either Drakeol or eicosane. The small hydrophobic surfactant, L31, and the 2 large hydrophilic surfactants, P103 and F108, did not. In fact, less protein was retained by the oil in emulsions containing these agents than in those that contained no surfactant at all.

TABLE II

Effect of pluronic polyols on stability and immunogenicity of emulsions

Surfactant[a]	HLB	Stability *in Vitro*[b]		Retention in Foot[c] Day 7	Antibody[d] titer Day 21	
		Day 0	Day 7		Antigen in oil	Antigen in PBS[e]
Drakeol (16C)-BSA						
		%	%	%		
L121	0.5	54	25	99	12 ± 0.5	1
L101	1.0	10	4	70	6 ± 0.9	0
L31	3.5	2	0	0	1 ± 0.6	NA[f]
P103	9.0	0	0	0	0	NA
F108	27	0	0	0	0	NA
None		5	0	0	0	NA
Eicosane (20C)-BSA						
L121		40	21	39	9 ± 1	0
L101		22	11	31	8 ± 0.5	0
L31		2	0	0	1 ± 0.6	NA
None		7	0	1	2 ± 0.2	NA
Drakeol (16C)-LBSA						
L121		20	12	52	7 ± 0.4	
L101		11	5	9	5 ± 1	
L31		2	0	3	1 ± 0.5	
P103		0	0	2	0	
F108		0	0	5	0	
None		9	0	2	0	
Eicosane (20 C)-LBSA						
L121		100	48	34	5 ± 0.9	
L101		91	55	41	4 ± 0.4	
L31		2	0	3	2 ± 0.7	
None		20	10	8	0	

[a] Oil-in-water emulsions were prepared by using the hydrocarbon, surfactant, and protein shown in PBS.

[b] The stability of the emulsion is the percent of the protein still attached to the oil drops after storage at 37°C immediately and 7 days after preparation.

[c] Groups of at least 4 ICR mice were injected in each rear footpad with 50 μl of the emulsion containing 25 μg of protein. The mean percent of the injected dose retained in the foot and popliteal lymph node at 7 days after injection are shown.

[d] Titers of antibody against BSA determined by passive hemagglutination and expressed as $\log_2$ of the reciprocal of the highest positive dilution of serum.

[e] Titers from animals immunized and bled similarly except that the BSA was added to the PBS rather than the oil phase of the emulsion.

[f] NA, not applicable because the surfactant did not hold BSA in the oil drops.

Equal doses of each emulsion were injected into the rear footpads of mice. The stability of the emulsions immediately after preparation, day 0, indicates the proportion of the labeled protein that was associated with oil drops at the time of injection. The rest was in solution in the PBS phase of the emulsion. The combinations of Drakeol or eicosane with either L101 or L121 promoted the retention of more ^{125}I-labeled BSA in the footpads than was predicted by the stability of the emulsions *in vitro*. The most effective combination, Drakeol-L121-BSA, caused 99% of the injected dose of BSA label to remain in the foot at 7 days, even though 46% of the labeled protein had already come off the oil drops by the time they were injected on day 0. When LBSA was substituted for BSA in this emulsion, less antigen was retained in the footpad even though more remained in the oil phase of the emulsion *in vitro*. L31, P103, and F108 were unable to prolong the retention of antigen label in animals' feet.

Immune response to antigens in emulsions. All animals immunized with BSA or LBSA in the oil phase of emulsions containing L101 or L121 produced substantial titers of antibody (Table II). The highest titers were produced by animals injected with Drakeol-L121-BSA. Those immunized with emulsions containing L31, P103, or F108 produced little or no detectable antibody. Low titers were produced by animals immunized with BSA in eicosane without a surfactant. As expected (12), LBSA was less effective in stimulating antibody formation than BSA. The time courses of the antibody response to the 2 most effective emulsions and to BSA in alum are shown in Figure 2. The peak antibody titers were reached about 6 wk after a single injection and persisted for several months. These experiments were repeated several times with modifications of dose or stain of animal. L121 was consistently more effective than L101 in stimulating antibody formation, and Drakeol was usually more effective than eicosane.

The physical form of the emulsions was important. In most experiments, lyophilized BSA or LBSA was homogenized with the hydrocarbon and surfactant before addition of PBS. As a control, some emulsions were prepared identically except that BSA was dissolved in the PBS rather than being mixed with the hydrocarbon and surfactant. As shown in the last column of Table II, these emulsions were unable to stimulate high antibody titers even though the amounts of each component injected were identical to those in the most effective preparations.

Histologic reactions. Animals injected with the various emulsions responded with different inflammatory reactions that correlated with the immune response produced. The emulsion that stimulated the highest antibody response, Drakeol-L121-BSA, caused severe swelling, redness, and tenderness of the feet, which began within hours after injection and persisted for several weeks. At 20 days after injection, the footpads of these animals measured 5.1 ± 0.2 mm compared with 3.1 ± 0.1 mm for uninjected controls. Histologically, these feet demonstrated an intense inflammatory reaction consisting predominantly of neutrophils. They also had a vasculitis characterized by neutrophil infiltration of vessel walls, degeneration of muscle in the media, and adherence of inflammatory cells to the intimal surfaces (Fig. 3*a*). Large areas of the feet underwent ischemic necrosis. The popliteal lymph nodes of these animals became enlarged with hyperplastic germinal centers and paracortical areas with numerous plasma cells (Fig. 4*a*). The feet of animals injected with BSA in L121-eicosane or L101 with either hydrocarbon showed similar but less intense reactions, which measured about 4 mm at 20 days. Emulsions containing L31,

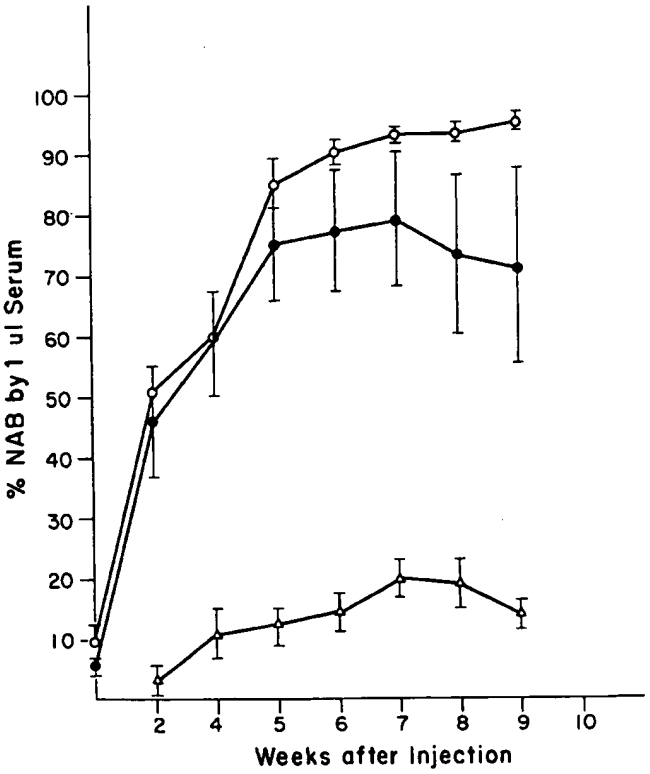


Figure 2. Serum antibody responses to emulsions containing BSA and L121. Serum antibody levels were determined by radioimmunoassay at weekly intervals after a single injection of 25 μg of BSA in each rear foot in 1 of 3 adjuvants (○—○, Drakeol-L121-BSA; ●—●, eicosane-L121-BSA; △—△, BSA in alum). The results are expressed as percent normalized antigen binding (% NAB) of 1 μg of ^{125}I-BSA by 1 μl of serum. The values for Drakeol-L121-BSA are higher than the linear range of this assay. When these sera were diluted, it was found that an average of 0.22 μl of sera from the 5-week bleeding bound 1 μg of antigen. At 8 weeks, 0.16 μl of serum bound the same amount of antigen. These results indicate that the animals produced several milligrams of specific antibody per milliliter of serum.

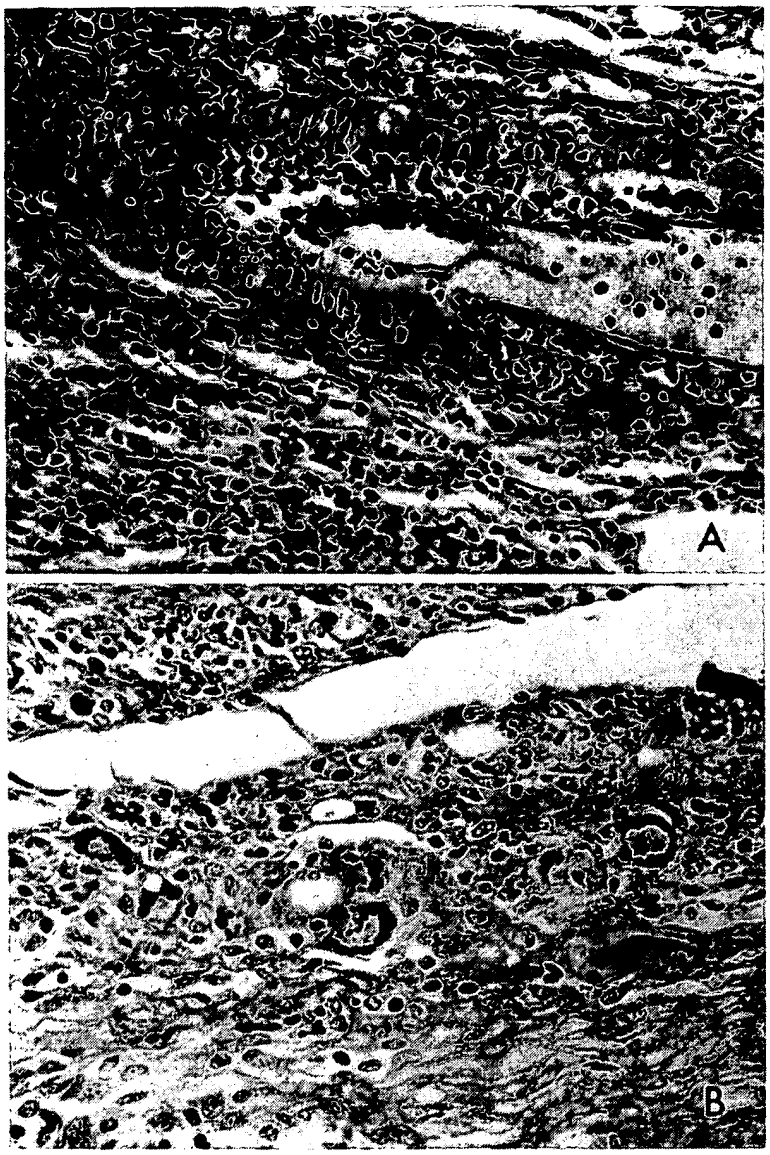


Figure 3. *A*, section of the foot of a mouse 7 days after injection of BSA-L121-Drakeol showing acute inflammation and vasculitis; *B*, section of foot of a mouse 7 days after injection of eicosane-L101-LBSA demonstrating a granulomatous reaction with multinucleated giant cells (H & E, × 400).

P103, or F108 caused very much milder inflammatory reactions. The popliteal lymph nodes of animals injected with emulsions containing L101 contained many fewer plasma cells than those injected with similar emulsions prepared with L121. However, they did have hyperplastic paracortical areas and occasional loosely formed granulomas. Emulsions prepared with LBSA caused more intense granulomatous reactions than those prepared with BSA. The emulsion eicosane-L101-LBSA caused the most intense granulomas, which contained many multinucleated giant cells (Fig. 3*b*). The lymph nodes of these animals also demonstrated granulomatous reactions with paracortical hyperplasia, very few plasma cells, and no germinal centers (Fig. 4*b*).

Another distinctive pattern of histologic change was seen in a portion of mice injected with emulsions containing LBSA and either L101 or L131. They developed a massive hypertrophy of their politeal lymph nodes within 4 days. These lymph nodes measured up to 0.8 cm in diameter and contained 1000-fold more paracortical tissue than normal lymph nodes. Histologically, they displayed a uniform pattern of sinus histiocytosis. The paracortical and medullary cords were filled with lymphocytes and the sinuses were packed with histiocytes. This lymph node hypertrophy appeared to result from recruitment of cells from other tissues in such numbers that the T cell areas of the animals' spleens were depleted.

Studies with cationic surfactants. Studies were done with the cationic surfactants, DDA and OCT, because their solubility and hydrophobicity are similar to the spreading agents, and they are known to be strong adjuvants (3). These agents promoted the retention of up to 76% of ^{125}I-labeled LBSA by hydrocarbon drops at 7 days *in vitro*, but were unable to cause the retention of more than 6% of an equal amount of BSA in the feet at that time (Table III). Nevertheless, the major patterns of retention of antigen observed with the Pluronic polys were also produced by these agents. Drakeol-DDA-BSA caused the retention of more antigen in the feet than was predicted by the stability of the emulsion *in vitro*. The opposite was true for emulsions containing LBSA. All of the emulsions caused acute inflammatory reactions with necrosis at the site of injection in the feet. The lymph nodes of animals injected with these emulsions developed paracortical hyperplasia with variable hyperplasia of other elements. Antibody titers after a single injection were low, but animals injected with emulsions containing DDA, and to a lesser extent OCT, were primed for a brisk secondary response to an injection of BSA in saline at 21 days.

DISCUSSION

In these studies, synthetic surface active agents, hydrocarbon vehicles, and chemically modified protein antigens were used to explore the relationships between the physicochemical properties

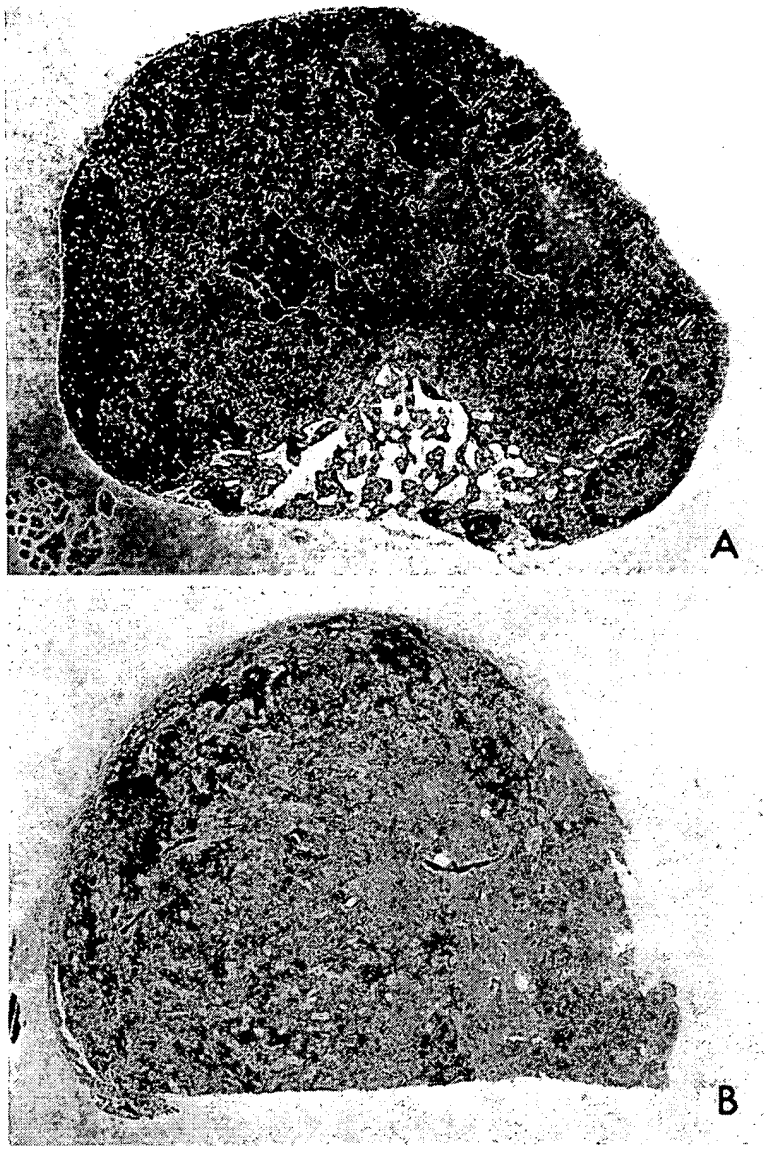


Figure 4. *A*, section of popliteal lymph node of mouse 3 weeks after injection of Drakeol-L121-BSA demonstrating paracortical and germinal center hyperplasia; *B*, section of the popliteal lymph node of a mouse 3 weeks after injection of eicosane-L101-LBSA demonstrating a granulomatous reaction in a hyperplastic paracortex (H & E. × 20).

of surface-active agents and their ability to serve as adjuvants. L121 was a powerful adjuvant in emulsions for stimulating antibody formation. L101 was less effective in stimulating antibody, but was more effective in inducing granulomas. The other Pluronic polyols had little activity for either. Since the Pluronic polyols are all composed of identical subunits, their different biologic activities cannot be explained by the presence of active sites on 1 molecule that are not present on all others. Likewise, Drakeol and eicosane are saturated hydrocarbons that differ only in the length of their carbon chains. Finally, even though lipid conjugation alters antigenic determinants on BSA, available evidence suggests that these alterations are responsible for only a minor portion of the changes in immunogenicity of LBSA (12, 17). We propose that the differences in the type and intensity of inflammatory and immune responses stimulated by the emulsions used in our studies were due to variation in their physicochemical properties. In particular, the activity of nonionic surfactants as adjuvants appears to depend upon a particular combination of physicochemical properties that allows them to focus biologically active molecules on hydrophobic surfaces. Several aspects of these experiments require discussion.

Previous studies of the adjuvant activity of oily emulsions have usually concentrated on their ability to form depots of antigen in tissue (18). Depots were generally assumed to be related to physical retention of antigen molecules by an insoluble matrix. Physical retention and slow release of antigen can quantitatively account for the adjuvant effects of Freund's incomplete adjuvant in some situations (18), but it cannot explain our results. The present experiments suggested that an oil-in-water emulsion must have a minimal level of stability *in vitro* in order for it to promote retention of antigen *in vivo*. However, some emulsions caused the retention of much more antigen label in the feet than could be accounted for by their stability *in vitro*. The inflammation and vasculitis in the feet of animals injected with Drakeol-L121-BSA apparently blocked lymphatic drainage from the feet so that nearly all of the BSA label was retained in the feet at 7 days, even though nearly half of it was in the PBS phase of the emulsion when it was injected. The protein was important in inducing this inflammation. The substitution of LBSA for BSA produced an emulsion that was twice as stable *in vitro*, but promoted the retention of only one-third as much antigen in the feet and stimulated much less inflammation. Eicosane-L101-LBSA caused granulomatous inflammation in the feet. The granulomas contained oil droplets and Langhans'-type giant cells, but there was less retention of antigen in the feet than observed in the oil *in vitro*. These results demonstrated that retention of antigen in tissues is a complex phenomenon that involves a variety of active host responses.

The intense neutrophil inflammatory response and germinal center hyperplasia caused by Drakeol-L121-BSA suggested that the chemotactic and other activities of complement (C) might be involved in the response to this emulsion. We found that hemolytic

TABLE III

Effect of cationic surfactants on the stability and immunogenicity of emulsions

Emulsion Components[a]			Stability		Retention in Foot Day 7	Antibody Titer[b]	
Hydrocarbon	Surfactant	Protein	Day 0	Day 7		Day 21 primary	Day 28 secondary
Eicosane	DDA	BSA	10	4.7	3	0	8 ± 1.2
Eicosane	OCT	BSA	1	0	1	1 ± 0.6	8 ± 0.6
Drakeol	DDA	BSA	2	0	6	2 ± 0.1	10 ± 2.1
Drakeol	OCT	BSA	2	0	1	0	5 ± 1.1
Eicosane	DDA	L-BSA	95	67	24	0	9 ± 2.7
Eicosane	OCT	L-BSA	90	76	15	0	7 ± 0.6
Drakeol	DDA	L-BSA	73	47	43	1 ± 0.2	8 ± 2.6

[a] Oil in water emulsions were prepared using the hydrocarbon, surfactant, and ^{125}I-labeled BSA or LBSA in 0.2% Tween 80 in PBS. Their stability and retention in feet and nodes were studied as described in Table II.

[b] Antibody titers on groups of at least 4 mice expressed as mean ± SE of $\log_2$ of the serum dilution were determined by passive hemagglutination on day 21. The animals were boosted with 25 μg of BSA in saline in each rear foot and the secondary titers determined on day 28.

guinea pig C was consumed by several emulsions containing either L121 or L101 as well as by LBSA alone (unpublished). Stark *et al.* (19) recently reported similar findings with lipid-conjugated proteins using mouse C. These results suggest that C is involved in inducing inflammatory reactions to these emulsions even before antibody is produced.

The physicochemical properties of surface-active agents with and without adjuvant activity offer clues to the mechanisms of their biologic activity. Several investigators have noted that surface-active adjuvants are larger and more hydrophobic than detergents. However, these properties have not been quantitated (2, 3). HLB is a particularly useful index of the physicochemical properties of nonionic surfactants that can be measured experimentally or approximated by calculation from molecular formulas. The two Pluronic polyols with adjuvant activity, L101 and L121, have very low HLB values. We calculated HLB values for a series of trehalose esters and other nonionic surface-active agents that have been studied for adjuvant activity. The trehalose esters are analogues of the mycobacterial glycolipid adjuvant, TDM. All of the agents with adjuvant activity have HLB values of less than 2 and would be classified as spreading agents (Table IV). None of the agents with HLB values greater than 2 were adjuvants. Spreading agents are insoluble in water and do not emulsify or solubilize lipids or membranes. Instead, they adhere to them and influence the interaction of soluble macromolecules with them. Although HLB values are not applicable to cationic surfactants such as DDA and OCT, these compounds have physicochemical properties similar to the nonionic spreading agents. Other cationic surfactants with long alkyl chains are adjuvants, whereas those with shorter alkyl chains and increased solubility in water have little or no activity (3).

Table IV also demonstrates a correlation between the ability of surface-active agents to promote retention of soluble macromolecules by oil drops and their activity as adjuvants. L121, L101, and TDM all require small oil drops for maximum expression of adjuvant activity. HLB determinations are able to identify surfactants that are large and hydrophobic enough to remain attached to oil drops and promote retention of proteins. We propose that these are the minimal properties of a compound that enable it to have this type of adjuvant activity. As illustrated by the experiments with DDA and OCT, the ability of a surfactant to promote retention of antigen by oil drops may depend on the properties of the antigen and the oil in addition to those of the surfactant. Nevertheless, it is most unlikely that a surfactant will be able to promote the retention of any protein by oil drops unless it has a low HLB. The surfactant in Freund's adjuvant, Arlacel A, has an HLB of 3.2, which allows it to stabilize water-in-oil emulsions so that antigen remains in the inner aqueous phase, but it is unable to hold antigen in isolated oil drops.

The nature of the molecular interactions between the surfactant and protein in adjuvant emulsions needs further study. The Pluronic polyols and other nonionic surfactants bind proteins (20). This binding and the strong lipophilicity of surfactants with HLB of less than 2 undoubtedly contribute to the retention of proteins by oil drops. The polar portions of the protein and surfactant molecules will preferentially localize on the surface of oil drops. If the protein was able to escape from the surface, it would be replaced by more protein from within until the entire drop was depleted. Consequently, a surfactant must be able to hold protein on the surface of oil drops if it is to hold it within them. In this situation, the local concentration of protein on the surface is several orders of magnitude greater than could be achieved by similar amounts of material in solution.

We propose that the ability to concentrate antigens and other materials on the surface of oil drops is an important component of the biologic activity of surface-active adjuvants. The oil provides a stable hydrophobic surface that anchors, orients, and concentrates the surfactant and adherent proteins so that they can more effectively activate inflammatory mechanisms and cells of the immune system. Depots of antigen and surfactant within the oil drop may serve to replenish its surface. Once an emulsion of the type used in these studies is injected into an animal, host proteins such as C or coagulation components will bind to the surfactant on the oil drops together with the antigen, and the entire complex will contact cells of the immune system. One would expect concentrated antigen presentation via this mechanism to be very different from that of the antigen alone.

Finally, some surface-active adjuvants, such as mycolic acid-conjugated MDP, probably have specific reactive sites. However, it should be noted that adjuvants whose activity is dependent on an ability to influence the presentation of other molecules do not need to react directly with cells and may not have any intrinsically reactive sites. This lack of intrinsic reactivity may explain the fact that some pluronic polyols are not adjuvants and the activity of others is critically dependent on the physical form of the emulsion in which they are injected. In studying these phenomena further, we believe that it will be important to consider that subtle variations in nonspecific physicochemical properties of adjuvants can produce qualitative as well as quantitative changes in immune responses. L101 and L102 are both spreading agents, but they differ in HLB, ability to reduce interfacial tension between oil and water, and other physiocochemical properties (21). The cationic surface-active agents vary in electric charge. We propose that the type and intensity of immune response stimulated by a particular emulsion is determined by the nature of the immunogenic determinants

TABLE IV

Correlation of HLB of nonionic surfactants with their adjuvant activity and ability to promote retention of immunogen by oil drops

Surfactant	HLB[a]	Strong Adjuvant[b]	Promote Retention of Immunogen by Oil[b]
Pluronic F108	27	No	No
Tween 80	15	No	No
Pluronic P103	9	No	No
Trehalose monobehenate	5.6	No	No
Trehalose dipalmitate	3.8	No	No
Pluronic L31	3.5	No	No
Arlacel A	3.2	No	No
Trehalose dibehenate	2.8	No	No
Maltose tetrapalmitate	2.0	Yes	ND[c]
Trehalose monomycolate	1.4	Yes	Yes
Trehalose dibehenylbehenate	1.4	Yes	Yes
Pluronic L101	1.0	Yes	Yes
TDM	0.7	Yes	Yes
Pluronic L121	0.5	Yes	Yes
DDA	NA	Yes	Yes
OCT	NA	Yes	Yes

[a] The HLB values for the Pluronic polyols, Tween 80, and Arlacel A were derived experimentally and obtained from the manufacturers' product literature. The values for the trehalose and maltose fatty acid esters are approximate values calculated from the structual formulas of the compounds by the method of Becher (10).

[b] The adjuvant activity and ability to promote retention of immunogens by oil drops of the Pluronic polyols, OCT and DDA, were derived from the present studies. The adjuvant activities of Tween 80, Arlacel A, DDA, and OCT were reported previously by Gall (3). The data for the trehalose esters were derived from McLaughlin *et al.* (22) and those on maltose tetrapalmitate from Nigam *et al.* (23).

[c] ND, not done; NA, not applicable.

presented on the surface of the oil drops, by the type of host proteins that adhere to the surface, and by conformational changes in them. The particular physicochemical properties of the surface-active adjuvant will markedly affect all of these functions.

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ACTIVATION OF HUMAN NEUTROPHILS WITH 1-*O*-HEXADECYL/OCTADECYL-2-ACETYL-*sn*-GLYCERYL-3-PHOSPHORYLCHOLINE (PLATELET ACTIVATING FACTOR)[1]

JAMES O. SHAW, R. NEAL PINCKARD, KATHLEEN S. FERRIGNI, LINDA M. McMANUS, AND DONALD J. HANAHAN

From the Departments of Medicine, Pathology, and Biochemistry, The University of Texas Health Science Center at San Antonio, and the Audie L. Murphy Memorial Veterans Hospital, San Antonio, TX 78284

1-*O*-Hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (AGEPC), the acetylated alkyl phosphoglyceride known as platelet-activating factor, stimulated human neutrophil (PMN) exocytosis, migration, superoxide production and aggregation over a concentration range of 10^{-10} to 10^{-5} M. AGEPC-induced PMN exocytosis of azurophilic (myeloperoxidase and β-glucuronidase) and specific (lactoferrin and lysozyme) lysosomal granules was rapid ($T_{1/2}$ = 20 sec), dependent on the presence of cytochalasin B, but was not associated with release of cytoplasmic LDH. As seen with the complement-derived peptide stimulus, C5a, AGEPC-initiated PMN enzyme release was dependent on temperature and cellular glycolysis but not on the presence of extracellular Ca^{++}. When analyzed by gradient analysis, PMN migration caused by AGEPC was primarily chemotactic in nature. An unusual feature for both enzyme secretion and migration was a decrease in response between 10^{-6} M and 10^{-5} M AGEPC. This decreased responsiveness could be explained by rapid PMN desensitization occurring at high AGEPC concentrations, limiting the overall cellular response. Rapid desensitization for exocytosis was demonstrated in PMN stimulated with AGEPC in the absence of cytochalasin B. When cytochalasin B was added sub-

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1) **Transforming growth factor-beta: A general view**
AUTHOR: Lawrence David A
AUTHOR ADDRESS: Growth Factors Group, UMR 146 du CNRS, Inst. Curie, Batiment 110, Centre Universitaire, 91405 Orsay, France**France
JOURNAL: European Cytokine Network 7 (3): p363-374 1996 **1996**
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2) **Gene therapy by** TGF **-beta-** receptor **-IgG** Fc chimera **inhibited extracellular matrix accumulation in experimental glomerulonephritis**
AUTHOR: Isaka Yoshitaka (Reprint); Akagi Yoshitaka; Kaneda Yasumi; Yamauchi Atushi; Orita Yoshimasa; Ueda Naohiko; Imai Enyu
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3) Hunter, 1981, J Immunol, 127 (3): 1244-?

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MINH TAM DAVIS
ART UNIT 1642, ROOM 3A24, MB 3C18
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REVIEW

Transforming growth factor-β: a general review

David A. Lawrence

Growth Factors Group, UMR 146 du CNRS, Institut Curie, Bâtiment 110, Centre Universitaire, 91405 Orsay, France.

ABSTRACT. Three isoforms of Transforming Growth Factor-β (TGF-β1, β2 and β3) exist in mammals. They play critical roles in growth regulation and development. Each isoform is encoded by a unique gene on different chromosomes. All three of these growth factors are secreted by most cell types, generally in a latent form, requiring activation before they can exert biological activity. This activation of latent TGF-β, which may involve plasmin, thrombospondin and possibly acidic microenvironments, appears to be a crucial regulatory step in controlling their effects. The TGF-betas possess three major activities: they inhibit proliferation of most cells, but can stimulate the growth of some mesenchymal cells; they exert immunosuppressive effects; and they enhance the formation of extracellular matrix. Two types of membrane receptors (type I and type II) possessing a serine / threonine kinase activity within their cytoplasmic domains are involved in signal transduction. Inhibition of growth by the TGF-betas stems from a blockage of the cell cycle in late G1 phase. Among the molecular participants concerned in G1-arrest are the Retinoblastoma (Rb) protein and members of the Cyclin/Cyclin-dependent kinase/Cyclin dependent kinase inhibitor families. In the intact organism the TGF-betas are involved in wound repair processes and in starting inflammatory reactions and then in their resolution. The latter effects of the TGF-betas derive in part from their chemotactic attraction of inflammatory cells and of fibroblasts. From gene knockout and from overexpression studies it has been shown that precise regulation of each isoform is essential for survival, at least in the long term. Several clinical applications for certain isoforms have already shown their efficacy and they have been implicated in numerous other pathological situations.

INTRODUCTION

It is now 15 years that Transforming Growth Factor-β (TGF-β) was discovered, independently, by two laboratories, those of Harold Moses and of Michael Sporn [1, 2], so before embarking on this review it is not without interest to mention briefly how this versatile molecule came to light. The major impetus leading to the discovery of TGF-β came during the 1970s from cell biology studies using mammalian and avian retroviruses (mostly capable of inducing sarcomas in animals). These studies had shown that retrovirally-transformed rodent fibroblasts secreted a substance which reversibly induced certain transformed cell parameters in untransformed cells [3]. This substance was called Sarcoma Growth Factor (SGF) and its biological activity was later shown to be due to a combination of two distinct molecules, TGF-α and TGF-β, acting in synergy [4]. TGF-α belongs to the EGF family of growth factor molecules and is not considered further here. It was rapidly established that TGF-β was secreted by cells transformed by other viruses or with chemicals, and more importantly, by normal cells and embryonic and adult tissues [5, 6]. These seminal studies, in addition to placing TGF-β in the expanding field of growth factor molecules, also lent support to the then newly proposed autocrine (and paracrine) concept [7] whereby cells respond to factors secreted by themselves (or by adjacent cells).

Over the intervening years three isoforms of TGF-β have been found to exist in mammals, of which the initially discovered TGF-β1 is now seen as the prototype of a much larger superfamily of sequence-related molecules involved in regulation of growth, differentiation and development [8]. The TGF-β super-family presently comprises over thirty distinct molecules found in a wide variety of species from Drosophila to Humans. In table 1 is shown a large sample of such molecules, some of which are known only by their cDNA. Hereon this review will focus on the three mammalian isoforms of TGF-β.

TGF-β ISOFORMS - STRUCTURE

All three mammalian isoforms, TGF-β1, β2 and β3, are 25kD homodimers (trace quantities of β1/β2 and β2/β3 heterodimers also exist) in their biologically active form [9, 10]. They show a high level of sequence conservation: thus β1/β2 have 74% amino acid identity, β2/β3 82% and β1/β3 78%. Each of

Table 1
The TGF-β super family

Protein	Host Species
founding members:	
TGF-β homodimers	
TGF-β1	mammals
TGF-β2	"
TGF-β3	"
TGF-β4 (homologue of mammalian TGF-β1)	chicken
TGF-β5	Xenopus
Inhibin family (homo/heterodimers; (one α, subunit, two β -βA & βB known)	
Inhibin A, (αβA)	mammals
Inhibin B, (αβB)	"
Activin A, (βAβA)	"
Activin AB, (βAβB)	"
DVR family (two phylogenetic branches, grouped by sequence similarity)	
nodal	mammals
BMP-2	"
BMP-4	"
DPP	Drosophila
BMP-5	mammals
BMP-6 (Vgr-1)	"
BMP-7 (OP-1)	"
BMP-8 (OP-2)	"
60A	Drosophila
Other more or less divergent members	
BMP-3	mammals
Vg 1	Xenopus
GDF-1	mammals
GDF-3 (Vgr-2)	"
GDNF	"
MIS	"

Abbreviations: DVR = decapentaplegic, vgr-1-related; DPP = decapentaplegic; Vg = vegetal pole; BMP = bone morphogenesis proteins; GDF = growth differentiation factor; OP = osteogenic protein; GDNF = glial-derived neurotrophic factor; MIS = Müllerian inhibitory substance

these homodimers is composed of two 112 amino acid chains, containing nine cysteine residues. X-ray diffraction studies of TGF-β2 crystals have revealed that of these nine cysteines, only one from each monomer (residue 77) is involved in forming an inter-chain disulfide bond linking the two monomers, whereas the other eight cysteines form intra-chain disulfide bonds [11, 12]. The latter studies also showed that TGF-β2 has an unusual 3-dimensional structure, vaguely ressembling an outstretched hand with only two fingers and a central cavity filled with four molecules of water. The 25kD form of TGF-β1, β2 and β3 represents the C-terminal end of a much longer precursor molecule (390 amino acids per monomer for β1 and 412 for β2 and β3) [9, 10]. Sequence conservation of the N-terminal part of these precursors is less than for the mature molecule and is

Table 2
Differences between the N-terminal part of TGF-β precursors

	TGF-β1	TGF-β2	TGF-β3
cysteine residues	3	6	5
glycosylation sites	3	3	4
RGD motif	yes	no	yes

evidenced by differences in the number of cysteine residues, glycosylation sites and by the presence or absence of the RGD motif (the latter is a recognition sequence for interactions with extracellular matrix molecules) (table 2). As expected for secreted molecules all three precursors have a 20-23 hydrophobic amino acid peptide signal sequence at the start of the N-terminus.

TGF-β GENES, mRNAs

The chromosomal locations of the human TGF-β genes are 19q13 for TGF-β1, 1q41 for TGF-β2 and 14q24 for TGF-β3 [13, 14]. Each of these genes comprises 7 exons, 6 introns (9). The corresponding mRNAs range from 2.5kb (TGF-β1) to 6.5kb (one of the TGF-β2 bands). It is indicative of a differential regulation of these three isoforms that their promoter sequences show distinguishing features between them [15, 16, 17]. Thus the TGF-β1 promoter lacks a TATA box but this element is present in TGF-β2 and β3; both TGF-β2 and β3 promoters possess a functional cyclic-AMP responsive element; and both TGF-β1 and β3 promoters are responsive to Sp1. The TGF-β1 promoter contains AP-1 sites and it has been shown that TGF-β1 autoinduction occurs via AP-1. In the 3' domain of the TGF-β1 gene an enhancer containing three potential phorbol ester responsive elements is present, suggesting that downstream elements may participate in transcriptional control of this gene as well as those found in the 5' promoter region [18].

TGF-β LATENCY

A little over 10 years ago it was discovered that most cells, normal, or transformed, and in particular human blood platelets, released TGF-β (isoform multiplicity was unknown at that time) as a high molecular weight complex in a biologically inactive form which was called latent TGF-β [19 - 22]. High molecular weight latent TGF-β could be converted to the active 25kD form following exposure to low pH (as well as alkalinisation, heating to approx. 100°C or treatment with urea). On the basis of these results it was proposed that activation of the latent form of TGF-β played a critical role in the regulation of its biological activity [22].

Biochemical analysis of latent TGF-β1 revealed that latency is conferred by a dimer of the N-terminal part of the TGF-β1 precursor non-covalently associated

with mature (25kD) TGF-β1 (23-25). This latency-conferring dimer is called β1-LAP, for latency associated peptide (Fig. 1). This structure of approx. 100kD is referred to as the small latent complex and in this form is secreted by numerous cultured cells, including CHO cells transfected by the TGF-β1 precursor cDNA. The transfection of TGF-β2 and β3 precursor cDNA into CHO cells also leads to secretion of small latent complexes of these two isoforms. β1-LAP contains three N-linked glycosylation sites and three cysteine residues. Two of these cysteines are critical for latency as they are involved in formation of a β-LAP homodimer: substitution of cys-223 and cys-225 by serine prevents dimerisation and results in secretion of directly active TGF-β1 [26]. Some cells produce a β1-LAP bearing mannose-6-phosphate (Man-6-P), a constituent which may be of importance as a binding epitope allowing latent TGF-β1 to home-in on Man-6-P/IGF-II membrane receptors [25]. An additional component called LTBP (for latent TGF-β binding protein), whose molecular weight is about 235kD, is present in the latent complex released from blood platelets, hence the designation large latent complex. LTBP is disulfide-linked to β1-LAP but is not necessary for latency [25]. Two other LTBP-like molecules have been cloned from human or murine sources, LTBP-2 and LTBP-3: typically these binding proteins contain two cysteine-rich motifs, namely EGF-like repeats and stretches of 8-cysteine residues. All three forms show homology to the fibrillin family of microfibrillar proteins [27, 28]. LTBP has been shown to facilitate secretion of latent TGF-β1 and may play a role in targeting the growth factor to extracellular matrices [25]. In addition a role for LTBP as a structural matrix protein in bone formation has been proposed [29].

The *in vivo* mechanism of latent TGF-β activation has received considerable attention ever since the latency phenomenon was discovered. On the basis of present knowledge it would seem likely that more than one mechanism is involved in such activation. *In vitro*, complete activation of all three latent TGF-β isoforms can be obtained by acidification to pH 3.0 [30]: since acidic microenvironments close to this pH exist *in vivo* (osteoclasts, activated macrophages, stroma of solid tumours), these may participate in the activation process as previously suggested [31]. However, more evidently physiological mechanisms have retained most interest. From co-culture experiments, where pericytes or smooth muscle cells were grown together with aortic endothelial cells, it was found that, while each cell type alone only produced latent TGF-β, the combination of both cell types led to *in situ* activation of the latent form [32, 33]. By the use of appropriate inhibitors and antibodies it was deduced that this activation was due to Plasmin. Retinoids also show a capacity to activate latent TGF-β, possibly by increasing the levels of Plasmin. Activation by co-culture and by retinoids appears to occur on the cell surface and may involve the participation of type II transglutaminase [34]. Thrombospondin-1 (TSP-1), a trimeric disulfide-linked glycoprotein, found in the

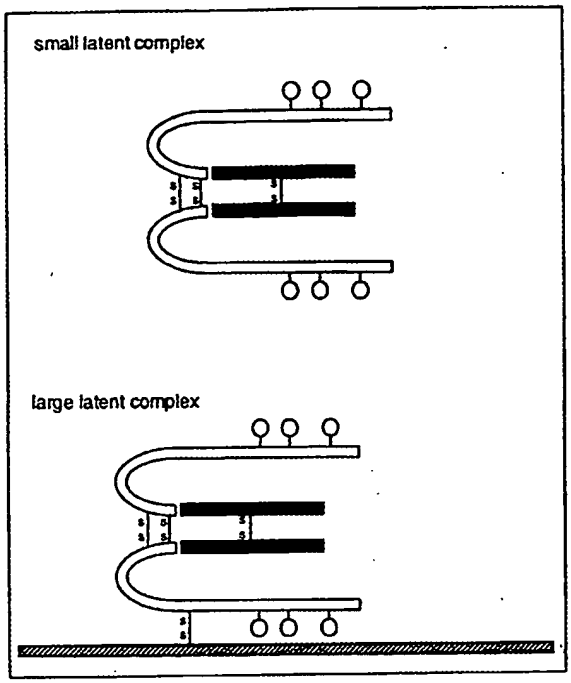

Figure 1
Diagram of the small and large latent TGF-β complexes
Curved empty lines represent terminal precursor (β1-LAP) filled stipple lines the mature 25kd dimer, the blobs indicate glycosylation sites, s-s indicate disulfide bonds (without regard to their number or position) filled hatched bar represents the Latent TGF-β Binding Protein (25).

α-granules of blood platelets, wound fluids and in extracellular matrices, activates latent TGF-β1 by a protease- and cell-independent mechanism [35]. TSP-1, of which other isotypes exist, is a multidomain protein containing characteristic repeat motifs. A tripeptide, *arg*, *phe*, *lys*, (RFK) present in the TSP-1 sequence has been found sufficient to activate latent TGF-β1, whereas a hexapeptide (GGWSHW) elsewhere in the TSP-1 molecule binds active TGF-β1 and prevents activation of the latent form by TSP-1 [36]. From the point of view of radiotherapy it is of great interest that active TGF-β1 was found in mouse mammary gland within one hour after exposure to ^{60}Co-γ radiation; this observation is compatible with the idea that locally active TGF-β1 may affect remodelling of stromal collagens in irradiated mammary gland [37].

Recombinant latent TGF-β1 has a half-life of about 90 minutes *in vivo*, whereas active TGF-β1 is removed from the circulation via the liver in 2-3 minutes [38]. This suggests that one function of latency is to provide stability and at the same time a locally activatable source of TGF-β1. The finding of significant levels (4ng/ml) of TGF-β1, but not of the two other isoforms, in human plasma suggest that TGF-β1 could play a hitherto unrealised endocrine role; in this study it was not possible to determine whether the plasma TGF-β1 was active or latent [38].

TGF-β RECEPTORS

Three major trans-membrane receptors and a number of soluble proteins are known to bind the TGF-betas [25]. As to the first category, type I (53kD) and type II (70 - 80kD) receptors are both serine/threonine kinases and appear to be preponderant in TGF-β signal transduction [39]. The currently published data indicate that the type II receptor is a constitutively active kinase, which autophosphorylates on ser/thr residues and possibly also on tyr and, upon binding ligand, transphosphorylates the type I receptor [40 - 45]. While some authors claim that type I receptors cannot autophosphorylate [40] and others claim the contrary [43], there is agreement that they do not transphosphorylate the type II receptor.

In any event the kinase activities of the intracellular domains of both type I and type II receptors are necessary for signalling downstream [39 - 45]. These receptors do not appear to undergo any notable ligand-induced downregulation [40]. As both receptors can exist as homodimers, it is thought that the functional complex may be a heterotetramer [42]. Two type II receptors have been identified [40, 41] and quite a family of type I receptors exist [39, 42]. Both the extra- and intracellular domains of type I receptors are shorter than their type II counterpart. In addition to a sequence of 3 cysteine residues just exterior to the transmembrane region in both types, the extracellular domain of type I receptors contains further upstream a cluster of 5 cysteines, this motif being absent in the type II receptor. A feature which distinguishes the intracellular domains is the presence in type I receptors of a SGSGSGLP sequence proximal to the kinase domain [39]. Although structurally similar, the type I and II receptors show less than 40% amino acid sequence identity in their kinase domains and less still in the other parts of these molecules.

In the much used mink lung epithelial cell model (CCL64) TGF-β-responsiveness depends on a particular type I receptor (ALK-5, also called R4 or TβRI), but in at least one other epithelial cell a different type I receptor (Tsk7L) appears to be involved [39]. Although the type I (ALK-5) receptor does not bind ligand in the absence of the type II receptor in CCL64 cells, it is conceivable that in other cells there exists a yet to be identified type I receptor capable of signalling without requiring type II receptor interaction. It appears then, at least in the CCL64 model, that ligand specificity depends on the type II receptor [39]. Both TGF-β1 and β3 show high affinity for the type II receptor, whereas TGF-B2 binds much less efficiently and via the intervention of type III receptor 39]. The latter receptor, usually referred to as Betaglycan [46], is the predominant one observed on gels in crosslinking studies as a long smear. Betaglycan is an approximately 300 kD proteoglycan containing heparin and chondroitin sulfate chains. Only the 100kD core protein, divested of these glycosaminoglycan chains, is required for binding to all three TGF-β isoforms; it has as yet no known enzymatic potential and seems not to be directly involved in signal transduction, other than by assisting presentation of ligand to receptor II [46]. A role in the stockage of ligand has also been evoked for Betaglycan [46]. The glucocorticoid analogue, Dexamethasone, enhances the expression of Betaglycan mRNA and increases binding of radiolabelled TGF-β1 to this receptor in osteoblast-like cells, suggesting an involvement of Betaglycan in glucocorticoid regulation of bone metabolism [47]. Some other membrane-bound TGF-β receptors have been found in particular cells (e.g., a class of isoform-specific receptors from rat glomeruli; and Endoglin, which binds TGF-β1 and β3 but not TGF-β2, shows domain and sequence relatedness to Betaglycan - except in the extracellular domains where their sequences are unrelated - and is highly expressed in endothelial cells, macrophages, pre-erythoblasts) [see ref. 25]. An important point concerning Endoglin is that its reduced form migrates on gels similarly to one of the type II receptors, so that cross-linking studies alone would not suffice to distinguish them in those cells expressing both of these molecules [48].

A lengthening list of soluble proteins have been shown to bind the TGF-betas [25], including a soluble form of Betaglycan composed of its extracellular domain [49]. Probably the earliest known soluble binding protein for TGF-β was α_2-Macroglobulin (α_2-M) [50]. The latter acts as a molecular trap for proteases, preventing their interaction with protein subtrates and binding to antibodies. α_2-M binds with 10-fold greater affinity to TGF-β2 than to TGF-β1 [51]. The TGF-β in human serum is associated with α_2-M in an inactive, latent form [50], which can be dissociated by Heparin [52] and this might explain why Heparin inhibits the growth of some cells.

Decorin, a proteoglycan containing chondroitin/dermatan sulfate chains, is able to bind all three isoforms of TGF-β [53], but the consequence of this binding on the biological activity of TGF-β depends on the system studied [54]. It appears that removal of the glycosaminoglycan chains enhances the ability of the Decorin core protein to bind TGF-β [53]. Latent recombinant TGF-β1 was found to be unable to bind to Decorin [53]. In a rat model of glomerulonephritis the administration of Decorin reduced extracellular matrix accumulation and attenuated disease symptoms, possibly by acting as a natural regulator of TGF-β activity [55].

In murine amniotic fluid α-fetoprotein (AFP) has been shown to bind TGF-β2, without loss of the growth factor's immunosuppressive activity [56]. As plasma levels of AFP increase during pregnancy and as TGF-β2 peaks around day 15 in mouse uterus, a complex of AFP/TGF-β2 might play a role in maternal tolerance of the fetal allograft. AFP plasma levels are also increased in some gastrointestinal, liver and testicular cancers and in cirrhosis and hepatitis [57], where it could act as a transporter of (at least) TGF-β2. It is also of interest that KCl treatment of human breast cancer cytosol revealed previously undetected amounts of

AFP [58]; the presence or not of a TGF-β-like molecule associated with AFP in this model might well be studied. Several other soluble proteins, fibronectin, β-amyloid precursor and thrombospondin (see section on Latent TGF-β) bind the TGF-betas, without inactivation of the latters' biological activity [25].

SIGNAL TRANSDUCTION OF TGF-β

More rapid progress in this area can be anticipated now that the two TGF-β receptors involved in signalling have been largely characterised (although one might leave open the possibility that other receptors, with perhaps somewhat different properties to those mentioned, are yet to be found in specific cell types). Some features of the signalling cascade may differ between the TGF-β isoforms given that TGF-β1 and β3 show a higher affinity than TGF-β2 at least for the first type II receptor cloned [39]. Furthermore, the myriad effects of TGF-β raise the idea that different signalling pathways could be involved in their transduction. In its inhibitory role TGF-β blocks the cell cycle late in G1, preventing passage into S phase [59].

Certain G proteins (Guanine nucleotide-binding proteins) have been implicated in this late G1 blockage [60]. Gi proteins, by inhibiting adenyl cyclase, influence cAMP levels and it has been shown that TGF-β1 reduces cAMP levels in some cells [61]. A critical element in TGF-β1-mediated signalling is the Rb tumour suppressor protein: TGF-β1 maintains Rb in an under-phosphorylated state, thereby preventing the cell cycle from continuing beyond G1 [62]. Among the molecular participants controlling Rb phosphorylation are cyclin D and E and their associated cyclin-dependant kinases, cdk-2, -4 and -6 [59]. By inhibition of the kinase activities of these cyclin E/cdk-2, cyclin D/cdk4 and cyclin D/cdk-6 complexes, the phosphorylation of Rb is suppressed in which state the latter acts as a cellular brake.

Several cdk inhibitors (cki) are known to reduce the synthesis or activity of these kinases e.g., p21-WAF, $p27^{kip1}$, $p15^{INK4}$ and $p16^{INK4B}$ [59, 63 - 67]. TGF-β1-mediated growth inhibition has been correlated with induction of these cki's and a reduction in the kinase activity of cdk-2 and/or cdk-4 [63 - 67]. Thus in cells growth-inhibited by TGF-β1, p21-WAF synthesis is induced and correlates with reduced kinase activity of cdk-2 [68]. Since p21-WAF was initially found as a p53-transactivated product, it is important to note that this TGF-β1-induction of p21-WAF can occur in the absence of a functional p53 protein [68].

One of the earliest examples of TGF-β1 stimulating monolayer cell growth was that of the mouse cell line AKR-2B. Here it was shown that growth stimulation was indirect, since TGF-β1 first induced c-*sis*, which codes for the mitogen PDGF-B and which secondarily stimulated proliferation [69]. Among several fibroblastic cell types growth-stimulated by TGF-β1 (and by the other isoforms) one can cite, Schwann cells [70], osteoblasts [71] and chondrocytes [72]. The human embryonic fibroblast WI38 (not a cell line, in that it has a finite number of population doublings) is stimulated by TGF-β1 [73], causing a drop in p21-WAF synthesis and an increase in cdk-2 kinase activity (68). Mouse NIH3T3 cells are also growth-stimulated by TGF-β1: here induction of c-myc occurs with the same kinetics as following serum addition, suggesting a more direct mitogenic effect of TGF-β1 [74].

More recently TGF-β1 was shown to increase the kinase activity of a member of the MAP kinase family called TAK1 in a mouse cell line [75]. In mammalian cells the links between the MAP kinase cascade and the more downstream cell cycle proteins await clarification so that the significance of TAK1 as a signalling intermediate remains unclear. A newly identified protein, TRIP-1, containing 5 WD domains, has been shown to specifically associate with the type II TGF-β receptor but not if the receptor kinase activity is destroyed, suggesting that autophosphorylation of the type II receptor might create a TRIP-1 binding site [76]. Interestingly, TRIP-1 and type II receptor were found to be coexpressed during mouse embryogenesis. Finally, the application of a new methodology has led to the identification of the preferred peptide sequence for the type I and II TGF-β receptors: in view of the different properties of these two receptor kinases, it was found, unexpectedly, that the preferred substrate for both receptors was a stretch of 6 lysine (KKKKKK) residues N-terminal to the phosphorylatable Serine [77]. This result should assist in identifying potential target proteins of the TGF-β receptor kinases.

BIOLOGICAL ACTIVITIES OF THE TGF-BETAS.

As numerous previous reviews have provided extensive coverage of the biological activities of the TGF-betas, this section will only briefly recall the major fields of activity in order to leave more space to describe the clinical implications of these molecules. *In vitro* the three mammalian isoforms of TGF-β, in their mature, activated forms, have similar activities, qualitatively, but show some quantitative differences which may depend on cell type [78]. However, from studies of the expression levels of the TGF-β isoforms during embryogenesis and histological analysis of organs and tissues from TGF-β1 and β3 knockout mice, there is now compelling evidence that each isoform has specific functions *in vivo* [79 - 81].

Essentially, the TGF-betas exert three biological activities, which are, at least in part, due to some overlapping effects [78]. Firstly, the TGF-betas inhibit the growth of most cells, though some mesenchymal cell types (Schwann cells, Osteoblasts, Chondrocytes, WI38 human fetal lung fibroblast) are growth-stimulated [68, 74, 78]. Secondly, they exert immunosuppressive effects, which derive to some extent from their anti-proliferative properties (e.g., inhibition of T- and B-lymphocytes). Thirdly, they enhance the deposition of extracellular matrix components (collagens, fibronectin, tenascin,

glycosaminoglycans and proteoglycans). It is constantly necessary to bear in mind the bifunctionality of the TGF-betas, not only as concerns cell proliferation, but also as regards immunosuppression (note that whereas immunoglobulin synthesis in general is reduced in B-lymphocytes, that of IgA synthesis is increased) [82] and matrix accumulation (thus, TGF-β1 can inhibit collagen type II synthesis in chicken chondrocytes) [83].

The bifunctional nature of TGF-β is further illustrated by the inversion of its effects on growth following viral infection: thus the anchored growth (adherent monolayers) of B-lymphocytes is normally growth-inhibited by TGF-β1, but is growth-stimulated when these cells carry Epstein-Barr virus [84, 85]; and the anchorage-independent growth (in soft agar) of Rat NRK-49F fibroblasts is stimulated by TGF-β1 but inhibited when these cells are transformed by Kirsten sarcoma virus [86]. In understanding the effects of the TGF-betas it is also useful to keep in mind that they may oppose the effects of other cytokines.

PATHOLOGICAL IMPLICATIONS AND CLINICAL APPLICATIONS OF THE TGF-BETAS.

The wide-ranging biological effects of the TGF-betas clearly suggest an essential role for these molecules in the regulation of cell growth and differentiation. Much evidence has now accumulated to suggest that dysfunction of TGF-β regulation (by overproduction or lack of one of the three isoforms, absence of receptors or inappropriate activation of the latent forms) can lead to a pathological situation. A large number of pathologies involve modifications of extracellular matrix, either in the initial triggering of a disease or in its aggravation, so that the enhancing effect of TGF-β on matrix formation is indicative of its playing a role in various fibrotic conditions. From gene knockout studies in mice it has been shown that TGF-β1 and β3 are essential for survival [79 - 81]: mice that lack TGF-β1 are born apparently normal but die within 3 weeks from cachexia due to a massive inflammation especially of the heart and lungs and those lacking TGF-β3 die rapidly, some 20 hours after birth, due to aberrant development of the lungs and palate. This last study implicates TGF-β3 specifically in the cleft palate defect. TGF-β3 has also been implicated in glucocorticoid-mediated acceleration of fetal lung type II cell maturation [87]. Transgenic mice which overproduce TGF-β1 also die just before or within hours after birth [88, 89]. Other transgenic mice have been obtained which overproduce TGF-β2 specifically in osteoblasts; these mice suffer from progressive bone loss and spontaneous fractures, resembling an osteoporosis-like phenotype [90]. Thus the absence or too much of the TGF-β isoforms is incompatible with, at least, long term survival.

A role for TGF-β1 in wound repair processes was indicated by its high concentration in blood platelets and from studies showing chemoattraction by TGF-β1 of monocytes, neutrophils and fibroblasts [78, 91].

Several reports have shown that local administration of TGF-β1 accelerates the healing of various kinds of cutaneous wounds [92]. However it has become evident that an excess of TGF-β1 causes the formation of scar tissue. It is known that wound repair in the fetus occurs without leaving scar tissue, but this is not so in the adult where scars can entail a functional handicap as well as the more obvious esthetic consequences [93]. During the course of evolution it appears that wound repair has been optimised to provide rapid healing, frequently in the absence of aseptic conditions, at the expense of scarring. Recent progress in this field suggests that by the use of antibodies against TGF-β1 and β2 and inclusion in the treatment protocol of TGF-β3, wound repair is enhanced without scar tissue formation [94]. The treatment cocktail and the precise timing of its application were critical in obtaining satisfactory healing. Following a penetrating wound to the brain, nerve regeneration fails due to the formation of fibrous scars: TGF-β1 is normally absent from the adult brain (at least in rodents), but is expressed after injury and appears to be responsible for scar tissue production [95]. If TGF-β1 synthesis could be modulated, this study opens the possibilty of manipulating the neuronal environment locally to allow nerve outgrowth after brain injury. For two other clinical disorders involving repair processes, venous ulcers and macular holes, the TGF-β2 isoform has been shown to improve these disorders significantly [96].

The TGF-betas have been implicated in several inflammatory diseases [91]. In patients with the inflammatory eye disease, uveitis, levels of active TGF-β2 were lower than in controls without intra-ocular inflammation [97]. Increased quantities of TGF-β1 have been found in synovial fluid from knee joints of patients with rheumatoid arthritis and intra-articular injection of TGF-β1 and β2 in rodents led rapidly to synovial erythema [91]. On the contrary, systemic administration of TGF-β1 or intra-articular injection of a neutralising antibody to TGF-β1 reversed synovial inflammation. These and similar studies indicate that localised administration of TGF-β1 enhances inflammation by increasing leukocyte adhesion and infiltration via chemoattraction of inflammatory cells, whereas systemic administration opposes this process probably because by this route TGF-β1 initially encounters capillary endothelium and decreases endothelial cell expression of adhesion molecules [91]. Thus, to exert its immunosuppressive functions TGF-β1 requires systemic delivery coupled with localised activation of the latent form. Nitric oxide (NO) produced from L-arginine is an important mediator of inflammatory events and is involved in neurotransmission, immunological tissue injury and host defence against bacteria [98, 99]. NO synthesis is stimulated by inflammatory cytokines like TNF-α, IL-1 and INF-γ. It has been shown that TGF-β1 suppresses NO formation in macrophages, bone marrow cells, cardiac myocytes, smooth muscle cells and retinal pigment epithelial cells, so it is likely that TGF-β1 could play a crucial role in controlling this inflammatory pathway [98, 99].

In view of the immunosuppressive and extracellular matrix stimulating activities of the TGF-betas, much research is focused on their role in the so-called auto-immune diseases [100, 101]. Several years ago it was reported that increased levels of TGF-β (unspecified isoform) were secreted by cultured blood cells derived from multiple sclerosis patients during regression of exacerbations [102]. More recent evidence in favour of TGF-β1 exerting an anti-inflammatory effect in such diseases comes from a report that TGF-β1 treatment of blood mononuclear cells taken from patients with multiple sclerosis (MS) or myasthenia gravis suppresses the autoantigen-induced mRNA expression of pro-inflammatory cytokines (IFN-γ, IL-4, IL-6, TNF-α and TNF-β) in these cells [103]. Since the TGF-β1 isoform is absent from the adult central nervous system (at least in rodents), [95], another TGF-β isoform is more likely to be clinically relevant in controlling these two diseases. Phase I clinical trials to test the effect of TGF-β2 treatment of MS patients were begun two years ago in Bethesda, USA [96].

As mentioned above, the overproduction or the degradation of extracellular matrix (ECM) constituents are known to be involved in many fibrotic diseases. An excess of TGF-β activity would bring about increased amounts of ECM, whereas a lack of such activity, together with a tumover of ECM components, would lead to their diminution. Excessive TGF-β1 activity has been shown to provoke glomerulonephritis in Rats and likely plays an unfavourable role in diabetic nephropathy [104]. Since TGF-β1 increases IgA production by B lymphocytes (in contrast to its action to decrease the synthesis of the other immunoglobulins), via the isotype switching phenomenon [82], it may also be involved in another kidney disorder, that of IgA nephropathy, in which IgA deposits occur in the glomerular mesangium. In the disease atherosclerosis, where lipid deposits are associated with fibrosis, apolipoprotein-a is a key factor which prevents the conversion of plasminogen into plasmin. This lack of plasmin leads to insufficient activation of latent TGF-β1 *in situ* and, in the absence of the growth-inhibitory action of the active form, allows proliferation of smooth muscle cells contributing to disease progression [105].

In the field of cancer, many reports have implicated a dysfunction of TGF-β regulation [78, 106]. Solid tumours require the formation of a vascularised stroma, without which the tumour cannot develop to a clinically deleterious size [107]. The TGF-betas stimulate production of stromal components such as fibronectin, tenascin and proteoglycans, so their over-expression would favour stroma formation. Such increased expression of the TGF-betas would also reduce the host immune response, allowing the tumour to escape from immunological elimination. The over-expression of TGF-β2 appears likely to be responsible for the immune deficiency in patients with Glioblastoma [108]. In other situations, those of some human colon (109) and gastric [110] cancer cell lines and in $CD4^+$ T cells [111] from patients with Sezary syndrome (a form of T-cell lymphoma), the absence of a functional type II TGF-β receptor has been implicated in the loss of growth inhibition. An interesting example involving the absence of all three TGF-β receptors is retinoblastoma [112]. The lack of, or inappropriate, activation of the latent forms of TGF-β are further possible mechanisms causing aberrant regulation of these growth factors. Thrombospondin, a stromal element, promotes angiogenesis and tumour invasion and these effects may be linked to its ability to activate latent TGF-β1 [36]. There is also evidence suggesting that the TGF-betas play a role in blood cancers: thus the impairment of the T-cell response and inhibition of lymphokine-activated killer cells in acute myeloblastic leukemia may be linked to uncontrolled expression of TGF-β1 and β2 [113]. During chemotherapy treatment of various cancers about 30% of patients develop oral mucositis: this severely aggravating condition can be controlled in hamsters by the application of TGF-β3 to the cheek mucosa [114]. The possibility of regulating the action of the TGF-betas with agents which induce their secretion such as retinoids, steroids (e.g., tamoxifen) and vitamin D derivatives [115], or with agents which could control their state of activation is the object of much current research and not only in the cancer field. Quercetin may be one such compound, in that it induces secretion of TGF-β1 by, and acts as a cytostatic agent on, leukemic blasts [116]. Clearly such manipulation would be of no avail in cases where there exists a receptor defect.

Finally, a mention is made of the role of TGF-β1 in moderating the consequences of myocardial [117] and cerebral [118] ischemia, of TGF-β1 as an aggravating factor in lowering immune response in the late phase of septic shock [119], of TGF-β2 in maternal immunity (prevention of fetus rejection) [120] and of an unspecified isoform of TGF-β in the development of oral tolerance [121] (this phenomenon has been successfully used in animal studies to treat certain auto-immune diseases).

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1) **Transforming growth factor-beta: A general view**
AUTHOR: Lawrence David A
AUTHOR ADDRESS: Growth Factors Group, UMR 146 du CNRS, Inst. Curie, Batiment 110, Centre Universitaire, 91405 Orsay, France**France
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2) **Gene therapy by** TGF **-beta-** receptor **-IgG** Fc chimera **inhibited extracellular matrix accumulation in experimental glomerulonephritis**
AUTHOR: Isaka Yoshitaka (Reprint); Akagi Yoshitaka; Kaneda Yasumi; Yamauchi Atushi; Orita Yoshimasa; Ueda Naohiko; Imai Enyu
AUTHOR ADDRESS: Osaka Univ., Osaka, Japan**Japan
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THANK YOU
MINH TAM DAVIS
ART UNIT 1642, ROOM 3A24, MB 3C18
272-0830

A2441 **S938* (MS)**

Gene Therapy by TGF-β-Receptor-IgG Fc Chimera Inhibited Extracellular Matrix Accumulation in Experimental Glomerulonephritis. Yoshitaka Isaka, Yoshitaka Akagi, Yasufumi Kaneda, Atushi Yamauchi, Yoshimasa Orita, Naohiko Ueda and Enyu Imai. *Osaka Univ., Osaka, Japan*
NAIST, Nara, Japan

Transforming growth factor-β (TGF-β) has been implicated in the pathogenesis of fibrotic diseases. The sustained overexpression of TGF-β is suggested to contribute to the development of glomerulosclerosis in human and experimental glomerulonephritis. Recent advance in cell biology, which revealed the complex regulation of TGF-β production and activity, has brought a number of possible approaches for inhibiting action of TGF-β. The soluble TGF-β type II receptor may inhibit the binding of TGF-β to its membrane receptor and may block its action. However, the monomeric soluble receptor has 10-times lower binding affinity than cell-surface receptor. Therefore we have generated a chimera cDNA encoding an extracellular domain of the type II TGF-β receptor and IgG-Fc, and have purified the chimeric soluble type II receptors. Then we tested whether the homodimers of the chimeric soluble type II receptor (TGF-βR-IgG) protein could competitively inhibit the biological activities of TGF-β *In vitro* and whether transfecting the expression vector of TGF-βR-IgG gene could suppress the ECM accumulation in anti-Thy-1 model of glomerulonephritis *in vivo*. In cultured BNul-7 cells, TGF-βR-IgG blocked TGF-β's inhibitory action on cell proliferation. In addition, TGF-βR-IgG completely inhibited the production of EDA+ fibronectin, which is induced by TGF-β, in cultured NRK cells exposed to TGF-β. Next we attempted to introduce the chimera cDNA into the muscle of the nephritic rats by HVJ-liposome mediated gene transfer method in order to block the TGF-β activity in nephritic glomeruli. The synthesized TGF-βR-IgG was effectively accumulated in the glomeruli through systemic delivery, and then TGF-β activity was significantly suppressed in the glomeruli from nephritic rats with a comparable effect in the reduction of ECM accumulation. In conclusion, TGF-βR-IgG successfully inhibit the action of TGF-β, and a gene therapy by chimeric TGF-βR-IgGFc might be feasible for the therapy of progressive glomerulosclerosis.

A2442

THY-1.1 Nephritis in Mongolian Gerbil. M. Ishizaki, M. Shimizu, K. Shichinoe, C. Kaminaga, H. Kitamura, Y. Masuda, Y. Sugisaki and N. Yamanaka (Intro. by F. Marumo). *Dept. of Pathology, Nippon Med. School, Tokyo, Japan and Dept. of Laboratory Animal Science, Nippon Med. School, Tokyo, Japan*

In a previous study, we showed that the thy-1.1 antigen exists in Mongolian gerbil (MG) and it was observed in the glomerular mesangial region the same as rats (*Biomedical Res* 10, 1989). Based on this observation, we attempted to induce thy-1.1 nephritis in MG. MGs were injected with 250 μg anti-thy-1.1 MAb (OX-7) or normal mouse serum (NMS) intravenously. After 20 minutes, they were injected with 0.5 ml normal Guinea pig serum (NGPS) as a complement substance. The animals were divided into three groups (G): G I (OX-7, n = 24), G II (OX-7 + NGPS, n = 24), G III (NMS + NGPS, n = 12). From each group 2–3 rats were euthanized at 30 minutes to 12 days. Kidney specimens were obtained and examined by LM, EM, and IF. In G I rats, infiltrating PMN cells were seen in the glomeruli at 1–3 hours, but mesangiolytic glomerular change and mesangio-proliferative change were not observed in all stages. In G II rats, infiltrating PMN cells were seen in the glomeruli at 1–3 hours and mesangiolytic change and mesangial proliferation were observed at 12 hours to 12 days.

In G III rats, glomerular structure was normal in all stages. Furthermore, we performed complement fixation test using MG serum and sensitized SRBC, and the result showed that MG serum did not have hemolytic activity.

The results of this study suggest that the early components of complement of MG serum were activated on OX-7 sensitized mesangium, but the terminal components were not activated on the mesangium and SRBC. NGPS complement was activated on OX-7 sensitized mesangium and successively induced mesangiolytic and mesangio-proliferative changes in MG.

In conclusion, the difference in thy-1.1 nephritis between MG and rat may be due to the differed complement activation. MG thy-1.1 nephritis model may be useful in studying the relationship between nephritis and complement.

A2443 **M920 (MS)**

The Number of Podocytes: A Predictor of Glomerular Sclerosis? Georg Jaremko, Ardan Patiwardan, Jens Nyengaard and Ann-Christine Eklöf (Intro. by B. Sundelin). *Karolinska Hosp., Dept. Woman & Child Health, Karolinska Inst., Physics IV, Royal Inst. of Technol. Stockholm Sweden and Stereol Lab, Univ. of Aarhus, Denmark*

Previous studies have shown that the Dahl salt sensitive (DS) rat kept on a normal salt diet develops glomerular sclerosis early in life even to a higher degree compared with Sprague Dawley (SPD) rats nephrectomized at 10 days of age. Progressive podocyte damage, as well as a reduced number of nephrons, have closely been linked to the development of glomerulosclerosis. In 6 weeks old male DS-rats (n = 7), the absolute number of podocytes per glomerulus (PC/Glm) was counted by confocal microscopy in propidium iodide/Lycifer Yellow stained and plastic embedded specimens. The total number of glomeruli per kidney (GlmNum) was estimated using the fractionator method. Weight matched male Dahl salt resistant (DR) and SPD rats (n = 7; both groups) served as controls. *Results:* (mean ± SD)

Group	PC/Glm	GlmNum	
DS	85±13	13344±1110	
DR	138±24#	17698±753*	*P < 0.001 *vs* DS
SPD	159±10*	21994±1278*	#P < 0.02 *vs* DS

Our data from the SPD rats relate closely to the average number of podocytes per glomerulus reported earlier in the SPD rat (Nagata & Kritz) when using a stereological method. Further, it was noticed that podocytes in glomeruli of DS rats with a low PC/Glm showed more pronounced degenerative changes compared with glomeruli with a higher number of podocytes. In some instances early sclerotic tuft lesions were noticed in glomeruli with low number of podocytes. *Conclusion:* Although the number of nephrons is of importance for the development of glomerulosclerosis our data suggest that the number of podocytes is a strong predictor of glomerulosclerosis.

A2444 **M826* (PD)**

How Pauci-Immune is ANCA-Associated Crescentic Glomerulonephritis (CGN)? J. Charles Jennette, Alice S. Wilkman, Robin H. Tuttle and Ronald J. Falk. *University of North Carolina, Chapel Hill, NC*

CGN with a paucity of immunoglobulins (Ig) by direct immunofluorescence microscopy (DIF) often is associated with antineutrophil cytoplasmic autoantibodies (ANCA). We determined the degree of DIF staining that distinguishes CGN that is likely to be ANCA-associated from CGN that is not. Renal biopsy and ANCA results from 213 patients with GN with crescents (excluding anti-GBM and lupus GN) were analyzed. The following table correlates the degree of glomerular DIF staining for Ig (expressed as the maximum staining for IgG, IgA or IgM) and dense deposits (DD) by electron microscopy (EM) with ANCA detected by indirect immunofluorescence (IIF) or enzyme immunoassay (EIA) for anti-proteinase 3 or anti-myeloperoxidase.

Bx Ig 0–4+**	ANCA in All CGN		ANCA in CGN with no DD		ANCA in CGN with DD	
DIF	IIF+	EIA+	IIF+	EIA+	IIF+	EIA+
0	**92%** 85/92	**85%** 78/92	**93%** 83/89	**85%** 76/89	**67%** 2/3	**67%** 2/3
0.5–1**	**82%** 59/72	**74%** 53/72	**86%** 56/65	**80%** 52/65	**43%** 3/7	**14%** 1/7
1.5–2	**53%** 10/19	**32%** 6/19	**100%** 5/5	**80%** 4/5	**36%** 5/14	**14%** 2/14
2.5–3	**35%** 6/17	**24%** 4/17	***	***	**35%** 6/17	**24%** 4/17
3.5–4	**8%** 1/13	**8%** 1/13	***	***	**8%** 1/13	**8%** 1/13

**Scored 0–4+ with 0.5+ increments, e.g. trace = 0.5+, 1–2+ = 1.5+,
*** No biopsies had > 2+ Ig staining with no DD by EM.

The frequency of ANCA is inversely proportional to the amount of Ig in glomeruli. At any level of DIF staining, the presence of EM DD reduces the likelihood of ANCA. When glomerular Ig is 2+ or less and there are no DD, the ANCA frequency is 90% by IIF (144/159) and 83% by EIA (132/159). We conclude that pauci-immune CGN should be defined as CGN with 2+ or less DIF staining for Ig and no DD by EM.

A2445 **T373 (PS)**

Expression of pp32, a Nuclear Phosphoprotein in the Normal and Diseased Kidney. S.S. Kadkol, T. Nadasdy, F. Kuhajda, L. Racusen and G.R. Pasternack. *Divisions of Renal and Molecular Pathology, Dept. of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD*

pp32 is a nuclear phosphoprotein expressed in self renewing and neoplastic cells. Paradoxically, pp32 inhibits oncogene induced transformation in vitro and confers resistance to programmed cell death, suggesting differential actions. We studied pp32 expression in paraffin embedded human kidney tissue by non isotopic mRNA in situ hybridization to identify and correlate pp32 expression with defined anatomical compartments in the normal kidney and to determine its expression in relationship to tubular regeneration in acute and chronic tubular injury. In normal kidneys (n = 6), moderately intense pp32 expression was observed in the epithelial lining of the distal nephron (thick ascending limb of Henle, distal convoluted tubule, collecting ducts) and focally in the visceral and parietal epithelial cells of the glomeruli. Proximal tubular epithelium, the interstitium and vasculature were negative. In contrast, in acute tubular necrosis (n = 11, [6 native and 5 transplant]) a weak to moderate focal pp32 expression was noted in the damaged/regenerating proximal tubular epithelium, and in the interstitial mononuclear inflammatory infiltrate, in addition to the distal nephron expression. In end-stage kidneys (n = 6), atrophic tubules showed a variable level of pp32 expression with the highest level in "classic" atrophic tubules, a moderate level in "endocrine type" tubules and a weak focal expression in "thyroid" tubules. Interstitial mononuclear inflammatory cells expressed pp32 strongly. Endothelial and myointimal cells of occasional arteries with intimal thickening showed pp32 expression focally.

Conclusion: pp32 expression is associated with regenerative activity in proximal tubules of the kidney. In the distal nephron segments, pp32 expression may have functions other than promoting regenerating since it is expressed in normal kidneys.

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(71) Applicant: CELTRIX PHARMACEUTICALS, INC. [US/US]; 3055 Patrick Henry Drive, Santa Clara, CA 95054-1815 (US).

(72) Inventors: SEGARINI, Patricia, R. ; 38 Devonshire Avenue, #5, Mountain View, CA 94043 (US). DASCH, James, R. ; 837 Seminole, Redwood City, CA 94062 (US). OLSEN, David, R. ; 276 Hedge Road, Menlo Park, CA 94025 (US). CARRILLO, Pedro, A. ; 1966 California Street, #7, San Francisco, CA 94109 (US).

(74) Agents: LUTHER, Barbara, J. et al.; Morrison & Foerster, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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(54) Title: USES OF TGF-β RECEPTOR FRAGMENT AS A THERAPEUTIC AGENT

(57) Abstract

A method of treating TGF-β excess is disclosed. The treatment is parenteral, oral or topical administration of TGF-β receptor fragment. Particularly effective is a soluble receptor fragment which resembles the extracellular portion of TGF-β binding protein II.

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USES OF TGF-β RECEPTOR FRAGMENT AS A THERAPEUTIC AGENT

DESCRIPTION

Technical Field

This invention relates to the fields of drug therapy and protein synthesis. A soluble TGF-β binding protein fragment is used to treat conditions characterized by an excess of TGF-β, including fibroproliferation and immunosuppression. The present invention also relates to recombinant expression of the binding protein fragment in prokaryotic and eukaryotic cells.

Technical Background

Transforming growth factor-β (TGF-β) represents a family of polypeptides, of which three are present in mammals, TGF-β1, TGF-β2 and TGF-β3. These factors have global effects on cell growth and differentiation (Roberts and Sporn (1990) Handbk. Exp. Pharm. 95:419-58). There is a growing body of evidence that TGF-β also modulates the immune process (Wahl et al. (1989) Immunol. Today 10:258-61). In addition to stimulating the congregation of immune cells at the site of injury, TGF-β also provides strong positive feedback for its own continued synthesis (Kim et al. (1990) Mol. Cell. Biol. 10:1492-1497). These factors have led to the investigation of the role of TGF-β in immune and fibroproliferative disorders.

In fibroproliferative diseases, TGF-β is becoming considered as a prominent factor. TGF-β is known 1) to stimulate cells to produce more proteins, including collagen, biglycan, decorin and fibronectin; and 2) to inhibit enzymes which degrade these proteins. Thus, TGF-β can cause fibrous tissue to accumulate. For example, in diabetic nephropathy and human mesangial proliferative glomerulonephritis, both fibroproliferative diseases, a prominent and important pathological feature is the accumulation of mesangial matrix (Mauer et al. (1984) J. Clin. Invest. 74: 1143-55). Likewise, postradiation fibrosis is characterized by excessive TGF-β, proliferation of fibroblasts and overproduction of connective tissue (Canney and Dean (199) Brit. J. Radiol. 63:620-23).

There have been several attempts to suppress the effects of TGF-β excess by administering antibody which is specific for TGF-β. In a pending patent application Serial No. 759,109, filed September 6, 1991, also assigned to Celtrix Pharmaceuticals, Inc., monoclonal antibodies to TGF-β were shown to have affinity constants ranging from 1.6 X 10^7 L/mol to 3.4 X 10^8 L/mol in a competitive radioimmunoassay test. These monoclonal antibodies were suggested for use in treating tumor cells that produce TGF-β to counteract the immunosuppressive effects of TGF-β. Another proposed use was treating metastatic cancers.

Border et al. (1990) Nature 346:371-74, found that antiserum against TGF-β suppressed experimentally induced glomerulonephritis, which was characterized by mesangial proliferation. Border et al. reported that the antibodies to TGF-β which were raised in rabbits had 50% binding to TGF-β at a ratio of 1:6000 in a radioimmunoassay. Antibodies typically have a molecular weight of at least 150 kilodaltons (kd).

More recently, Border et al. ((1992) Kidney Int. 41:566-570) mentioned that "[o]ther investigators have used our strategy of antagonizing TGF-β by administering anti-TGF-β *in vivo*. This approach has confirmed a causal role for TGF-β in pathological matrix accumulation by reducing scar formation in fetal skin, in the central nervous system following wounding and reduction of interstitial fibrosis in a model of acute lung injury." (p. 567) In a paper accepted for publication in J. Exp. Med., Wahl also cites TGF-β excess in "a spectrum of connective tissue disorders including rheumatoid arthritis, scleroderma, myelofibrosis, and hepatic, intraocular, and pulmonary fibrosis."

Anti-TGF-β antibodies have been administered to animals given an intraarticular injection of bacterial cell walls in an amount sufficient to cause the development of arthritis. One intraarticular injection of anti-TGF-β was sufficient to prevent arthritis. (Wahl (1992) J. Clin. Immunol. 12:1-14). In the J. Exp. Med. paper, Wahl et al. reported that antibody injected into a joint before systemic administration of streptococcal cell wall (SCW) resulted in a 75% decrease in joint inflammation. Even if the antibody were injected about two weeks after the SCW injection, at which time the inflammation had become chronic, there was still a significant benefit. Likewise, Goddard et al. ((1990) Cytokine 2: 149-55) found that TGF-β inhibited the growth of cultured synovial cells, which was reversed by administration of neutralizing antibodies.

TGF-β-specific antibodies also were injected into the margins of healing dermal wounds in adult rats. Control wounds (those with irrelevant antibody or TGF-β) all had scarring, but the antibody-treated wounds healed completely with normal strength but no scar formation. Shah et al., (1992) The Lancet 339: 213-14.

TGF-β-specific antibody has also been found to partially restore, at least in laboratory tests, the defective T-cell responses due to excess TGF-β production such as in patients with acquired immune deficiency syndrome (AIDS). (Kekow et al. (1990) Proc. Natl. Acad. Sci. 87:8321).

In rat prostate cancer, TGF-β1 is overexpressed, compared to normal prostate tissue. It appears that TGF-β1 enhances tumor growth by stimulating tumor cells (Steiner and Barrack (1992) Mol. Endocrinol. 6:15-25). Steiner and Barrack tested the effect of anti-TGF-β antibody on over-producing prostate cancer cells which had stopped growing. The antibody caused the prostate cancer cells to begin proliferating again. In mouse prostate cancer, both TGF-β1 and 3 were elevated and were correlated with progression to malignancy and may even promote carcinoma (Merz et al. (1991) Mol. Endocrinol. 5:503-13).

Another way of suppressing TGF-β in experimental glomerulonephritis in rats, which is associated with TGF-β1 excess, was a low-protein diet. Both the excreted nitrogen and the expressed TGF-β1 decreased. (Okuda et al. (1991) Proc. Natl. Acad. Sci. U.S.A., 88:9765-69.)

In addition, TGF-β has natural inhibitors, including decorin and endoglin. Yamaguchi et al. ((1990) Nature 346:281-84) have proposed that decorin binds TGF-β and provides negative regulation of the TGF-β by interfering with TGF-β binding to a receptor. Decorin is highly glycosylated and has a molecular weight of about 46 kd and an affinity for TGF-β of about 10^{-8} to 10^{-9} M.

Endoglin also appears to bind TGF-β with an affinity constant of about 5 X 10^{-11} M. Endoglin also is highly glycosylated and has a molecular weight of about 90 kd.

Previously, anti-TGF-β, decorin and endoglin have been suggested as inhibitors of TGF-β. However, these three proteins share the undesirable feature of high molecular weight (20-180 kd). Affinity constants for the antibody and decorin are relatively low, ranging from about 10^{-8} to 10^{-9} M. Moreover, administering antibodies from other species can cause cross-species reactions.

An improved inhibitor would have a much lower molecular weight and higher affinity. This combination of features would permit much lower doses and increase ease of administration. Moreover, a native protein would not cause cross-species reactions.

Nine classes of TGF-β receptors have been found. The main receptors on cells are Type I, Type II and Type III. A soluble form of the Type III receptor has been detected, and it binds TGF-β similarly to the membrane-bound Type III receptor (Andres et al. (1989) J. Cell. Biol. 109:3137-45).

The human Type II receptor has been cloned and codes for a protein of 563 amino acids (Lin et al. (1992) Cell, 68: 775-85). This protein contains three discreet domains: a 136-amino acid extracellular domain, a 30-amino acid hydrophobic transmembrane domain, and a 376-amino acid intracellular domain. The extracellular region binds TGF-β. The Type II receptor has a very high affinity for TGF-β, on the order of about 10^{-11} to 10^{-12} M. Furthermore, the Type II receptor is a native human protein, which should help avoid cross-species reactions. And finally, the region which binds TGF-β is only about 100 amino acids long. Therefore, its molecular weight is only a fraction of previously suggested inhibitors.

Production of recombinant heterologous proteins in prokaryotic host cells is essential to produce

commercially feasible amounts of protein. Unfortunately, bacterial host cells such as *E. coli* often are not ideal or simply cannot be used to produce such proteins. This is because the proteins, when over-expressed, form refractile, insoluble "inclusion bodies" and/or prove lethal to the cells. Inclusion bodies are found in the cytoplasm of the cell. Although inclusion bodies can be isolated from the cell by cell lysis and centrifugation, subsequent purification of the proteins involves dissolving the inclusion bodies and renaturing the proteins. Renaturation is not always effective or efficient. A variety of mechanisms have been sought to overcome these problems. However, none of the methods are ideal.

Purification of proteins produced in bacterial host cells has also proven to be problematic. In many cases, the proteins of interest, particularly when incorporated into inclusion bodies, co-purify with bacterial cell wall components which can be toxic.

Mammalian cells are sometimes preferred for recombinant production because they can appropriately glycosylate and properly fold proteins.

Disclosure of the Invention

In one embodiment, the invention provides a method for treating an individual for a medical condition associated with TGF-β excess. The method provides for the parenteral, oral or local administration of a sufficient amount of TGF-β-binding receptor fragment to the individual to reduce excess TGF-β activity in the individual.

In another embodiment, the method of the present invention provides for the administration of a fragment of human recombinant TGF-β receptor.

In yet another embodiment, the method of the present invention provides for TGF-β receptor fragment administration by intravenous, intraocular, intraarticular, transdermal and enteral methods.

In another embodiment, the method of the present invention provides for the administration of the Type II TGF-β receptor. In another embodiment, the administered protein is a fragment of Type II TGF-β receptor. In a further embodiment, the administered protein is Type I TGF-β receptor; in another embodiment, the protein is a Type I TGF-β receptor fragment. In yet another embodiment, Type III TGF-β receptor fragment is administered.

In another embodiment, the TGF-β receptor fragment is administered to patients with cancer. In further embodiments, the type of cancer is plasmacytoma, glioblastoma, or prostatic or ovarian carcinoma.

In another embodiment of the present invention, the TGF-β receptor fragment is administered to patients with collagen vascular diseases, such as systemic sclerosis, polymyositis, scleroderma, dermatomyositis, or systemic lupus erythematosus.

In another embodiment of the present invention, the TGF-β receptor fragment is administered to patients with fibroproliferative disorders. In a further embodiment, the TGF-β receptor fragment is administered to patients with hepatic, intraocular and pulmonary fibrosis. In a further embodiment, the TGF-β receptor fragment is administered to patients with diabetic nephropathy, glomerulonephritis, proliferative vitreoretinopathy, rheumatoid arthritis, liver cirrhosis, and biliary fibrosis.

In still another embodiment, the method of the present invention provides for treating a wound in an individual to avoid excessive connective tissue formation

which is associated with TGF-β excess. The method provides for administration of a sufficient amount of TGF-β-binding receptor fragment to the individual to reduce the excess of TGF-β in the individual. In further embodiments, the types of wounds include surgical incisions, trauma-induced lacerations and wounds involving the peritoneum for which the excessive connective tissue formation is abdominal adhesions. In a further embodiment, the excessive connective tissue formations which are avoided include scars, including those where the scar involves restenosis of blood vessels, and hypertrophic scars, and keloids.

In another embodiment of the present invention, the method provides for administration of TGF-β receptor fragment in the condition of TGF-β excess characterized by immunosuppression associated with an infectious disease. In a further embodiment, the immunosuppression may be associated with trypanosomal infection or viral infections such as human immunosuppression virus, human T cell lymphotropic virus (HTLV-1), lymphocytic choriomeningitis virus and hepatitis.

In another embodiment, the invention provides a method of increasing the effectiveness of a vaccine. In this aspect, TGF-β-binding receptor fragment is administered to an individual about to receive a vaccine or receiving a vaccine. The amount of TGF-β-binding receptor fragment is sufficient to increase the individual's immune response to the vaccine. In a preferred embodiment, the vaccinated individual is immunocompromised.

In another embodiment, the invention provides a method of preventing postradiation fibrosis in an individual undergoing or about to undergo radiation therapy. TGF-β-binding receptor fragment is administered

in an amount sufficient to prevent excessive fibrous tissue formation.

Brief Description of the Drawings

Figure 1 is a photograph which illustrates binding of biotinylated TGF-β2 to filter paper embedded with Type II soluble TGF-β receptor fragment (sβ-RII).

Figures 2A and 2B are photographs of electrophoresis results showing that sβ-RII from *E. coli* and COS cells, respectively, binds with specificity to TGF-β in solution.

Figures 3A and 3B show the reaction of antibody specific for a fragment of the sβ-RII with *E. coli* soluble and inclusion body fractions (Figure 3a) and with COS supernatants (Figure 3b) transfected with control vector (right lane) or sβ-RII vector (left lane).

Modes For Carrying Out the Invention

The following terms are used herein:

"Individual" means a living organism, including humans, other mammals and any other animals which produce TGF-β.

"TGF-β" is a family of peptide growth factors, including five members, numbered 1 through 5.

"TGF-β excess" as used herein is an amount of TGF-β present in serum or tissue which is significantly above the normal level. More preferably, TGF-β excess is a level between about 2 and about 20 times normal. Even more preferably, TGF-β excess is a level between about 2 and about 15 times normal. For example, Deguchi measured 24-hour TGF-β production of bronchoalveolar cells and reported normal levels of 410 ± 225 pg/10^7 cells against excess TGF-β production of 1288 ± 453 pg/10^7 cells in

systemic lupus erythematosus and 1417 ± 471 pg/10^7 cells in scleroderma ((1992) Ann. Rheum. Dis. 51:362-65). TGF-β excess can be determined, in combination with normal levels, by measurement of the TGF-β protein, of TGF-β MRNA, or of products whose synthesis is stimulated by TGF-β, such as collagen.

TGF-β receptors are cell surface proteins, of which three (Type I, Type II and Type III) are known in mammals.

TGF-β receptor Type II is a membrane-bound protein with an intracellular domain, transmembrane domain and extracellular portion which binds to TGF-β. Human TGF-β receptor Type II has been determined to have the amino acid sequence shown in Lin et al., 1992, and corrected by personal communication as shown in SEQ ID NO:1.

A TGF-β receptor fragment is a portion or all of a TGF-β receptor molecule which is capable of binding TGF-β. Preferably, this fragment has a high affinity for TGF-β. Even more preferably, the TGF-β receptor fragment has a greater affinity for TGF-β than does anti-TGF-β antibody or decorin.

"sβ-RII" refers to protein fragments of the extracellular portion of the TGF-β receptor Type II which are soluble and bind with high affinity to TGF-β. Preferably, the affinity is in the range of about 10^{-11} to 10^{-12} M, although the affinity may vary considerably with fragments of different sizes, ranging from 10^{-7} to 10^{-13} M. These fragments are proteins consisting of about 136 amino acids or less. Most preferably, sβ-RII is about 136 amino acids.

In another embodiment, sβ-RII is about 10-110 amino acids in length and comprises the TGF-β binding site. Preferably, the sβ-RII of this embodiment is a protein of about 50-80 amino acids.

If the entire native amino acid sequence of 136 amino acids is used, the amino acid sequence resembles that of the entire extracellular portion of the Type II receptor. When smaller sβ-RII fragments are employed, they resemble various portions of the extracellular portions of the Type II TGF-β receptor, so long as they bind TGF-β with high affinity.

Although the sequence of sβ-RII is based on the native TGF-β receptor II extracellular fragment, the definition of sβ-RII also comprises analogs of sβ-RII which have high affinity for TGF-β. Such analogs include those made by conservative substitutions of amino acids, as well as those made by mutated cells synthesizing sβ-RII. Only analogs with high affinity for TGF-β are included in this definition.

"Connective tissue" is fibrous tissue characterized by the presence of fibroblasts and fibrous proteins such as collagen and elastin.

A "therapeutic composition" as used herein is defined as comprising sβ-RII and other physiologically compatible ingredients. The therapeutic composition may contain excipients such as water, minerals and carriers such as protein.

"A sufficient amount of TGF-β-binding receptor fragment" as used herein refers to the amount of TGF-β receptor fragment that neutralizes the biologic activity of excess TGF-β. It may be determined by (1) suitable clinical variables of improvement, (2) pathologic evaluation of the effects on fibrosis and/or immunosuppression or prevention of fibrosis, or (3) a direct inhibition of TGF-β.

This invention provides for administering to an individual with a medical condition associated with TGF-β excess a sufficient amount of TGF-β-binding receptor fragment, such as sβ-RII, to reduce excess TGF-β activity

in the individual. The TGF-β-binding receptor fragment is all or only a portion of a receptor which is capable of binding TGF-β. sβ-RII is made by synthesizing the extracellular domain of the TGF-β Type II receptor (β-RII) and developing a fragment of this β-RII domain as a high affinity, soluble binding protein (sβ-RII) for TGF-β. This invention further provides for delivering sβ-RII to a site where TGF-β is in excess, such as in disease states characterized by fibroproliferation and immunosuppression such as is associated with infectious disease.

The sβ-RII of the present invention may be synthesized by means known in the art. The longer, 136-amino acid version is preferably synthesized by recombinant techniques which are known to those in the art. Alternately, and preferably for shorter versions of sβ-RII, sβ-RII also can be synthesized by solid-phase synthetic methods known to those in the art.

While not wishing to be bound by any particular theory, it appears that the sβ-RII regulates TGF-β activity by competing for TGF-β with cell-surface receptors. It is further believed that sβ-RII inactivates TGF-β by removing it from the free pool of TGF-β available to interact with cell surface receptors. Depending on the pharmacologic properties of clearance, the sβ-RII/TGF-β complex is removed from the site of TGF-β excess. This complexing with excess TGF-β reduces the amount of free TGF-β. With less TGF-β available to complex with cell receptors, TGF-β induced fibroproliferation slows down, resulting in stasis of the disease state.

The sβ-RII fragments of the present invention may be used to treat viral infections in which there is an overproduction of TGF-β and immunosuppression. Examples of viruses with which TGF-β excess is associated

include, but are not limited to, hepatitis C, lymphocytic choriomeningitis, human immunodeficiency virus (HIV), and human T cell lymphotropic virus (HTLV-1), the latter being discussed in Kim et al. ((1991) Mol. Cell. Biol. 11:5222-28).

The sβ-RII fragment of the present invention may be used to treat the trypanosome-mediated immunosuppression. This may be caused by Trypanosoma cruzi or Leishmania cruzi, among others. Immunosuppression in Leishmania cruzi has been studied by Barral-Netto et al., (1992) Science 257:545-48.

The sβ-RII of the present invention also may be used to increase the efficacy of vaccines. Because TGF-β may cause immunosuppression, the administration of sβ-RII can counteract immunosuppression caused by TGF-β and increase the vaccine recipient's immune response to the vaccine. sβ-RII should be particularly effective in immunosuppressed patients. sβ-RII may be administered before or concomitantly with the vaccine.

The sβ-RII fragments of the present invention also may be used to treat forms of cancer which are associated with excess TGF-β. It is also generally known that TGF-β is produced by different tumor cells (sarcomas and carcinomas). Specific examples of tumors in which TGF-β production is excessive include glioblastoma (Wrann et al. (1987) EMBO. J. 6: 1633-36; and Bodner et al. (1989) J. Immunol. 143: 3222-29) and plasmacytoma (Berg and Lynch, (1991) J. Immunol. 146: 2865-72). This TGF-β production may protect the tumor cells from recognition by the host's immune system, Wrann et al. (1987) EMBO. J. 6:1633. In these situations, TGF-β suppresses the proliferation of T and B cells, NK cells, LAK cells, and macrophages that are normally involved in tumor destruction. The sβ-RII fragments of the present invention also may be used to treat prostatic cancer.

Merz et al. ((1991) Mol. Endocrin. 5:503-13) reported that elevated TGF-β is correlated with progression of prostatic hypertrophy to malignancy and to metastasis. Steiner and Barrack have also reported that overproduction of TGF-β1 prostatic tumors produced more extensive metastatic disease ((1992) Mol. Endocrin. 6:15-25)). Therefore, early treatment with sβ-RII may help abort progression to malignancy; and later treatment may prevent metastasis.

The administration of sβ-RII fragments of the present invention may be used in fibroproliferative disorders. As mentioned above, animal models of glomerulonephritis have shown good results with anti-TGF-β antibodies blocking excess TGF-β. These antibodies will be difficult to deliver because they have a high molecular weight and they may result in severe allergic reactions when they are derived from other species. Thus, it would be preferable to administer a lower molecular weight, native protein or close analog, such as sβ-RII, in glomerulonephritis. Kidney diseases associated with TGF-β excess include, but are not limited to, mesangial proliferative glomerulonephritis, crescentic glomerulonephritis, diabetic nephropathy, renal interstitial fibrosis, renal fibrosis in transplant patients receiving cyclosporin, and HIV-associated nephropathy. These conditions are associated with excessive fibrous tissue formation which administration of sβ-RII should suppress.

As sβ-RII by itself is not known to have an effect aside from capturing TGF-β, sβ-RII may safely be administered during or at the end of retinal reattachment surgery, which is the most common cause of proliferative vitreoretinopathy (PVR) (Connor et al. (1989) J. Clin. Invest. 83:1661-1666).

Another important fibroproliferative condition is rheumatoid arthritis (RA), which is also associated with excess TGF-β production. Data indicate that blocking TGF-β at any time in the development or chronic stages of RA may help stop the progressive deterioration of the joint and bone. Hence, sβ-RII fragments of the present invention may be administered to patients with early joint pain and to patients with prolonged joint pain and deteriorated joints. The current theory is that joint deterioration in RA is due to an overproduction of TGF-β. Excess TGF-β has been measured in joints after test animals are injected with streptococcal cell walls, whose presence is believed to cause RA. Because anti-TGF-β antibody blocks arthritic changes in this model, it is believed that sβ-RII may also have a positive effect.

Work in an animal model suggests that chronic liver cirrhosis which is characterized by excess collagen deposition, could be mediated by TGF-β. (Czaja et al. (1989) J. Cell. Biol., 108: 2477-82; and Hoyt et al. (1988) J. Pharm. Exp. Ther. 246: 765). In patients with chronic hepatitis and cirrhosis, the levels of TGF-β1 MRNA were 2-14 times higher and correlated with higher measurements of serum procollagen than were observed in patients with normal or fatty livers. Six of eight patients with hepatitis C were treated with alpha-interferon for one year and had sustained clinical improvement and normalization of serum procollagen activity. These treated patients also had normal levels of TGF-β1 mRNA in liver biopsy specimens taken at the end of one year, further supporting the role of TGF-β in liver fibrosis (Castilla et al. (1991) N. Engl. J. Med. 324:933-40).

Cirrhosis of the liver is a widespread condition which is associated with an abnormally high degree of fibrous tissue in the liver and frequently with

high levels of TGF-β. Cirrhosis is the end product of the liver's reaction to many types of injury, including alcohol abuse, exposure to other chemicals, infections (such as hepatitis), intestinal bypass operations and others. In cirrhosis, normal hepatocytes, which produce fibrous blood proteins and clear toxins from the blood, have been replaced by fibrous tissue. In many instances, TGF-β is in excess. In such instances, the sβ-RII fragment of the present invention may be used to treat cirrhosis.

sβ-RII fragments also may be used to treat biliary cirrhosis, a condition in which the bile ducts become scarred and interfere with the gall bladder emptying its enzymes and digestive juices into the small intestine and hence with the digestion of fats. sβ-RII fragments also may be of assistance in treating this condition when it is associated with excess TGF-β.

Other conditions associated with excess TGF-β levels include idiopathic pulmonary fibrosis and myelofibrosis. To complex with excess TGF-β and to slow the development of excess fibrous tissue, sβ-RII is intended for administration in these conditions.

The sβ-RII fragments of the present invention may be used to treat collagen vascular diseases that are associated with overproduction of TGF-β. It is currently believed that there is an overproduction of TGF-β in collagen vascular diseases, such as progressive systemic sclerosis (PSS), polymyositis, scleroderma, dermatomyositis, eosinophilic fascitis, and morphea. Collagen vascular diseases may also be associated with the occurrence of Raynaud's syndrome. Among other effects, excess TGF-β production may also be involved in interstitial pulmonary fibrosis, an end-stage lung disease which is associated with autoimmune diseases such as systemic lupus erythematosus (SLE) and scleroderma

(Deguchi, (1992) Ann. Rheum. Dis. 51:362-65); or it may be caused by chemical contact, allergies to dust and hay fever. A therapeutically effective amount of the sβ-RII of this invention may be administered to neutralize the biologic activity of excess TGF-β, which in turn would prevent unwanted fibrosis.

sβ-RII fragments of the present invention also may be used in preventing excessive scarring in patients who are known to form keloids or hypertrophic scars. sβ-RII may be administered to prevent scarring or excessive scarring during healing of various types of wounds including surgical incisions and traumatic lacerations. sβ-RII may be applied to skin wounds before they are closed to help in healing without scar formation. sβ-RII also may be placed in surgical abdominal wounds to help prevent adhesion formation which occurs all too commonly after that type of surgery. Williams et al. ((1992) J. Surg. Res. 52:65-70) recently reported that TGF-β was more effective in promoting postoperative peritoneal adhesions than a control of diluent. Intraperitoneal injections of TGF-β for five days did not induce adhesions in unoperated rats. Williams et al. proposed that preventing TGF-β production postoperatively might help prevent adhesion formation. Rather than preventing TGF-β production, which could have systemic side effects, the present invention provides for the local administration of sufficient Sβ-RII to complex with local TGF-β overproduction and prevent excessive healing processes.

According to Lindholm et al., ((1992) J. Cell. Biol. 117:395-400), TGF-β1 is a strong inhibitor of astrocyte proliferation and may thus interfere with nerve regeneration. Because sβ-RII complexes with TGF-β, sβ-RII may encourage nerve regeneration. In deeper wounds

where nerves are cut, the application of sβ-RII also can help nerve regeneration.

TGF-β excess also has been reported in nasal polyposis, a condition characterized by multiple polyps (Ohno et al. (1992) J. Clin. Invest. 89: 1662-68). sβ-RII can help lower the TGF-β excess and slow the hyperproliferation that results in polyps. sβ-RII can be administered after polyp surgery to prevent excessive scarring and recurrence of polyps. sβ-RII can also be administered to inhibit polyp formation in the intestine.

sβ-RII may also be administered following coronary angioplasty, preferably placed along the inside of the affected arteries. According to Karas et al., ((1991) Clin. Cardiol. 14:791-801) restenosis or scarring and reclosing of arteries following coronary angioplasty is seen in approximately one-third of patients operated on. Because the fibrous network which ultimately develops into a scar normally accumulates rapidly, early administration of sβ-RII would reduce excess TGF-β in this area and slow excessive proliferation of connective tissue and restenosis.

TGF-β excess has also been observed in cardiac fibrosis after infarction and in hypertensive vasculopathy. To aid in proper healing without excess scar or fibrous tissue formation, sβ-RII can be administered in these conditions.

TGF-β excess also has been observed in the tissues of patients receiving radiation therapy. Such tissue is characterized by excess connective tissue development, epithelial thinning and blood vessel occlusion associated with overgrowth of endothelial cells. Administration of sβ-RII will complex with the excess TGF-β and will contribute to healing without excessive fibrosis.

Formulation, Administration and Dosage

The formulation, method of administration and dosage sβ-RII will depend upon the disorder to be treated, and the medical history of the patient. These factors are readily determinable in the course of therapy. Suitable patients with conditions caused by an excess of TGF-β can be identified by laboratory tests, medical history and physical findings. TGF-β excess can be determined directly by immunoassay (Example 6 below) of the patient's serum or of the affected tissue. Excess TGF-β can also be determined by bioassays such as the cell proliferation assay described in Kekow et al., (1990) Proc. Natl. Acad. Sci. U.S.A. 87: 8321-25. Excess TGF-β also can be determined indirectly by measuring the level of TGF-β mRNA (for example, in the polymerase chain reaction of Kekow et al.).

The medical history may reveal facts which support a diagnosis of fibroproliferative disorder, collagen vascular disease, immunosuppression, or of potential for problematic wound healing, as in peritoneal adhesions following surgery, or restenosis of blood vessels after coronary angioplasty. Conditions which are identified as being associated with high levels of TGF-β and/or proliferation of fibrous tissue are considered to cause TGF-β excess.

Patients may have a wide spectrum of physical findings which are indicative of such disorders. Skin biopsies have been used to test TGF-β in patients with systemic sclerosis. Swollen, hot joints are seen in arthritis.

In accordance with the method of the present invention, the formulation comprises sβ-RII in an administrable form. The method of the present invention provides for formulating sβ-RII in modes which are readily apparent to those skilled in the art.

Preferably, the sβ-RII is dissolved in physiologically compatible carriers.

Physiologically compatible carriers for sβ-RII include intravenous solutions, such as normal saline, serum albumin, 5% dextrose, plasma preparations, other protein-containing solutions and TPN solutions. The preferred carrier for parenteral administration of sβ-RII is a sterile, isotonic aqueous solution, such as saline or 5% dextrose. Even more preferred is normal saline with human serum albumin. For use in enhancing the immune response to vaccines, sβ-RII may be mixed with the vaccine formulation.

Depending on the mode of administration, the sβ-RII composition may be in the form of liquid or semi-solid dosage preparations, such as for example, liquids, suspensions or the like. Alternatively, a solution of sβ-RII may be placed into an implant, such as an osmotic pump, for the slow release of sβ-RII over an extended period of time. Alternatively, sβ-RII may be provided in sustained release carrier formulations such as semi-permeable polymer carriers in the form of suppositories or microcapsules. See, for instance, U.S. Patent No. 3,773,919 for Microcapsular Sustained Release Matrices Including Polylactides; Sidmon et al., Biopolymers 22 (1), 547-556 (1983) for copolymers of L-glutamic acid and γ-ethyl-L-glutamate; Langer et al., J. Biomed. Res. 15, 167-277 (1981) for poly(2-hydroxyethylmethacrylate) or the like. Finally, receptor fragmentation and modifications, such as fusion of the sβ-RII fragment with human immunoglobulin (IgG) or with polyethylene glycol (PEG) so as to extend the half life of the sβ-RII fragment, are other alternative forms of administration.

The mode of administration delivers sβ-RII to the individual in a safe, physiologically effective manner. sβ-RII may be given by intraocular, intranasal,

subcutaneous, intravenous, intramuscular, intradermal, intraperitoneal, intraarticular, enteral or other conventional routes of administration.

In a preferred embodiment, the sβ-RII of the invention is administered locally to the affected tissue sites by bolus injection or perfusion. For example, for PVR, the preferred mode of administration is a single intraocular injection. Local administration is also preferred in peritoneal wounds to avoid adhesion formation and in other wounds to encourage healing with no keloids or visible scars. For nasal polyposis, nasal drops are preferred.

Local and systemic administration are equally preferred in lung fibrosis (parenteral injection or nasal spray or drops) and cancer. In early, localized tumors, localized administration may be preferred. In later tumor stages, where cancer cells may have metastasized, parenteral administration may be preferred, alone or in combination with local injection. RA can be treated by intraarticular or systemic administration.

Systemic administration is the preferred mode of administration in glomerulonephritis, liver cirrhosis, immunosuppressive conditions (such as viral infections, AIDS and trypanosomal infections), and in widespread skin diseases (such as progressive systemic sclerosis, diffuse fascitis, and generalized morphea). Systemic administration also is preferred when sβ-RII is used to enhance vaccine response. sβ-RII can be administered with the vaccine by subcutaneous, intramuscular or intradermal injection.

The dose of sβ-RII to be administered can be readily determined by those skilled in the art, based on the usual patient symptoms discussed above. The dosage of sβ-RII to be given in a bolus injection is preferred to be between 20 ng and 300 mg. The bolus injection may

be repeated over several days, or the sβ-RII can be continuously infused. If given as an intravenous infusion, the amount of sβ-RII to be infused over a 24-hour period is about 1 mg to about 100 mg.

The amount of sβ-RII to administer may also be determined by maintaining the local tissue concentration of TGF-β at a subnormal level, or about 1-1,000 μg/ml. For tumors, the amount administered is preferably about 20 ng to 300 mg sβ-RII per gram tumor tissue.

Preferably, sβ-RII is applied topically, injected at the site of the problem or injected intravenously. Most preferably, sβ-RII is administered by bolus injection at the site where TGF-β is to be controlled. By intravenous injection, sβ-RII should be administered at a rate to maintain a circulating serum concentration sufficient to reduce the TGF-β excess.

Preferably, the patient is started with a relatively low dose of sβ-RII. The low dose preferably should be continued until the patient's acute phase is ameliorated or adequately improved, as indicated appropriate physical findings and laboratory results. Such improvement may be evident in two to three weeks. In the absence of significant improvement, the dose of sβ-RII should be increased.

For patients to be vaccinated, the dose of sβ-RII is preferably between 20 ng and 300 mg. Preferably, more sβ-RII is given to immunocompromised vaccinated patients. sβ-RII can be administered a short time before the vaccine, to permit sβ-RII to complex with TGF-β prior to vaccination. Or sβ-RII can be administered simultaneously with the vaccine.

The invention has been disclosed by direct description. The following examples show that the sβ-RII binding protein fragment can treat conditions

characterized by an excess of TGF-β; however, these examples should not be taken in any way as limiting the scope of the method.

EXAMPLES

Example 1

sβ-RII was expressed in *E. coli* as a 15 kd protein of 136 amino acids with no carbohydrate. Those skilled in the art are familiar with cloning genes in the fashion detailed in Lin et al. ((1992) Cell 68: 775-785) Lin et al. also disclose the complete amino acid and nucleotide sequences.

The expression vectors used in this work were prepared from pET3b supplied by W. Studier. A new vector, pETX, was prepared and differs from pET3b in that it contains a modified oligonucleotide linker downstream of the unique BamHI site in the vector, having the sequence 5'...GGATCCCGTGGAGGATTAAACCATGGATGGATGCATAAGCTT CGAATTC...3' (SEQ ID NO:2).

In addition, the restriction fragment between the unique EcoRI site and the EcoRV site downstream of the terminator was deleted so that both restriction sites were destroyed.

The pDJ12833 vector backbone was derived from pETX by reconstituting the tetracycline resistance gene and inserting a 385 bp fragment carrying the par locus of pSC101 (according to the method of Meacock and Cohen, (1980), Cell 20: 529-42) into the unique PvuII site of pBR322 backbone present in pET3b, the parent vector. pER10088 is similar to pDJ12833 but does not carry the par locus. Both vectors contain, in addition, the translational coupler described in Squires, et al. (1988) J. Biol. Chem. 263:16297-302.

The DNA encoding the extracellular domain of the type II receptor was subcloned after PCR amplification of the 136 codons of that domain from a pre-existing cDNA clone, pH2-3FF (obtained from MIT). The oligonucleotides used in the amplification were

5'...GGGGATCCGATAGTGGAGGATGATTAAATGATCCCACCGCACGTTCAGAAGT ...3' (5' oligo) (SEQ ID NO:3); and 5'GGGGAATTCAAGCTTAGTCAGGATTGCTGGTGT TATATTCTTCTGA...3' (3' oligo) (SEQ ID NO:4). Amplification was for 40 cycles with annealing at 55°C. The single major product of this amplification was purified using the Mermaid Kit from Bio 101 (La Jolla, CA) and digested with BamHI and EcoRI, gel purified and ligated to pETX vector digested with the same enzymes + calf alkaline phosphatase. After transformation of JM109, the structure of a single recombinant plasmid (pDJ16902) was confirmed by DNA sequencing.

pDJ16919 was constructed by transferring the expression cassette from pDJ16902 into the backbone of expression plasmid pDJ12833.

The sβ-RII protein was obtained by growing *E. coli* strain W3110 DE3 containing pDJ16919 at 37°C in a Biostat E fermentor (manufactured by Braun) and inducing sβ-RII synthesis. The cell paste was collected and stored at -80° C until ready for use.

An aliquot of paste was suspended in 0.1 M Tris pH 8.0, 5 mM EDTA, 1 mM PMSF. Lysozyme was added to a final concentration of 0.2 mg/ml and the sample incubated at 4°C for approximately 30 min. The sample was sonicated with 3-60 sec pulses on ice on a Branson 250 Sonifier and centrifuged at 10,000 rpm on a Sorvall (Wilmington, DE) centrifuge. The pellet was collected, and this fraction is known as the "inclusion body fraction".

The inclusion body fraction was solubilized in 6 M guanidine HCl and dialyzed against 10 mM HCl. The dialyzate was neutralized with 1M NaOH and chromatographed on a Q-Sepharose column (Pharmacia, Piscataway, NJ). The bound material was eluted with a salt gradient (0-0.5 M sodium chloride) in 0.1 M Tris pH

7.5 and the fractions were analyzed on 18% SDS-PAGE with Coomassie blue stain. The bulk of the sβ-RII appeared in fractions 5-15 and was pooled for analysis or further purification.

The sβ-RII protein prepared in this example was found to bind TGF-β when sβ-RII was bound to a hydrophobic support, as discussed in Example 2, and in solution as discussed in Example 3.

Example 2

sβ-RII had been previously solubilized from inclusion bodies in 6M guanidium hydrochloride with 25mM dithiothreitol. The sample was diluted five fold and split into two fractions. The "control" sample was left untreated. The "Sβ-RII" sample was brought to a final concentration of 5mM cystamine and incubated overnight at 4°C. Both samples were dialyzed against 0.1M Tris, concentrated, and applied to a membrane for a ligand blotting assay as follows. First, Immobilon P membrane (Millipore Corp., Bedford MA) was soaked in methanol for five seconds and then in tris-buffered saline (50 mM Tris, pH 7.5, 0.2 NaCl)(TBS) for one to ten minutes. Meanwhile, the dot-blot apparatus (Gibco-BRL, Gaithersburg, MD) was set up and the membrane inserted on top of a sheet of 3MM paper (Whatman International, Ltd., Maidstone, UK) wetted with TBS. This assembly was tightened.

Fifty μl TBS was placed in each well. Next, samples of SβRII and control were added in any volume up to 200 μl and vacuum filtered very slowly for about 5-15 minutes.

The assembly was disassembled, the filter was marked and blocked in TBS with 5% dried milk overnight at 4° C. It is important to start with a fresh TBS-milk

suspension.. Alternately, the filter could also be blocked for one hour at room temperature.

One blot was incubated with 50 pM biotinylated TGF-β2 (1.25 ng/ml) alone while the other blot was incubated with 50 pM biotinylated TGF-β2 and 50 nM TGF-β2(+) as a competing ligand for 1.5 hours at 37°C. Next the filter was washed three times, 10 minutes each with TBS/0.05% Tween 20. Next streptavidin-HRP (Zymed Laboratories, Inc., South San Francisco CA) was added at a dilution of 1:1000 in TBS/5% milk/0.05% Tween 20. This was incubated at room temperature for 30 minutes. The filter was washed three times, 10 minutes each with TBS/0.05% Tween 20.

The filter was moved to a new dish. It was overlaid with about 6 ml of a 1:1 mixture of ECL reagents (Amersham Corp., Arlington Heights, IL) and incubated for one minute.

The filter paper was blotted on a paper towel and was placed in a plastic bag and sealed. The filter was exposed to film (XAR-5, Eastman Kodak, Rochester, NY) for one to 45 minutes.

The results of the sβ-RII fragment binding to biotinylated TGF-β2 bound to filter paper are shown in Figure 1.

Example 3

This example demonstrates that sβ-RII binds with TGF-β in solution. Q-Sepharose bound material (804 μg/ml) was incubated overnight at 4°C with 50 pM ^{125}I-TGF-β1 without (-) or with (+) 40 nM unlabeled TGF-β1. The complexes were cross-linked at 4°C for 15 min with 0.3 mM disuccinimidyl suberate and electrophoresed under reduced conditions on 18% SDS-PAGE. The gel was dried and complexes were visualized by autoradiography. Figure 2A is a photograph of these results. Molecular size

standards are indicated at the left in kilodaltons. The "*" indicates monomeric TGF-β1; the "**" indicates dimeric TGF-β1; the "→ " indicates the appropriate size complex at approximately 31 kD (monomer of TGF-β cross-linked to sβ-RII).

Example 4

Plasmid H2-3FF containing a 4.5 kb cDNA fragment of the human TGF-β type II receptor (Lin et al., (1992) Cell, 68:775-85) was cloned into the EcoRI site of TGF-β plasmid pcDNAI was digested with EcoRI. The 4.5 kb cDNA fragment was isolated and subcloned into the EcoRI site of plasmid BlueScript SKII$^+$. The plasmid obtained from this subcloning experiment was named BS/βRII RI. This plasmid was used to transform *E. coli* strain CJ236 to obtain single-stranded DNA containing uracil residues (UssDNA). Single-stranded uracil-containing DNA was isolated by infecting CJ236 cells containing plasmid BS/βRII RI with helper phage VCS-M13 and subsequent kanamycin selection. The UssDNA obtained from these cultures was to be used as a template for site-directed mutagenesis experiments.

An oligonucleotide was synthesized with the antisense sequence of the TGF-β type II receptor from nucleotides 553-583 (nucleotides are numbered according to Lin et al., 1992) with the exception of the codon for Asn^{106} which was changed such that a stop codon would be inserted. The sequence of the oligonucleotide used to create this mutation is 5'-TAGCAACAAGTCAGGTTAGCTGGTGTTATATTC-3' (SEQ ID NO:5). This primer in combination with the UssDNA described above was used to carry out an *in vitro* mutagenesis experiment (Kunkel et al., (1985) Proc. Natl. Acad. Sci. USA 82:488-492). Clones containing the desired mutation were identified by nucleotide sequencing. A clone

containing a stop codon in place of Asn^{106} was named BS/βRIIs.

BS/βRIIs plasmid DNA was purified and digested with EcoRI and BgIII to isolate a 1177 base pair fragment comprising the 5' untranslated sequence and sequences for the extracellular domain (ECD) of the receptor and a portion of the transmembrane domain containing the stop codon created by site-directed mutagenesis. This 1177 base pair EcoRI-BgIII fragment was subcloned into the EcoRI-BgIII site of plasmid pSG5 (Stratagene). This plasmid allows expression of heterologous genes in mammalian cells utilizing a SV_{40} early promoter and SV_{40} splice and polyadenylation signals. The plasmid created by this subcloning experiment was called pSG/βRIIs.

Example 5

COS-M6 cells were maintained in DMEM high glucose media supplemented with 10% fetal bovine serum and antibiotics. COS-M6 cells were transiently transfected using the DEAE-dextran method as described by Seed et al. (1987) Proc. Natl. Acad. Sci. USA 84:3365-3369. Briefly, plasmids pSG/βRIIs or pSG5 (negative control) were complexed with DEAE-dextran and added to the cultures for 2 hours. Following this incubation, the cells were glycerol shocked, washed and then allowed to recover for 32 hours. The cultures were then washed three times with serum-free media and allowed to grow for an additional 72 hours in serum-free media. The media were collected, the cell debris was removed by centrifugation and then analyzed for the presence of soluble TGF-β type II receptor expression.

Example 6

Initially the soluble type II receptor protein (sβ-RII) was detected by visualization on a Western blot.

Supernatants were electrophoresed under reducing conditions on SDS-PAGE and blotted (Towbin et al., (1979) Proc. Natl. Acad. Sci. USA 76:4350-4354). Polyclonal rabbit antisera prepared against a peptide (residues 68-89) were incubated with the blot and the immunoreactive proteins were visualized after developing with an alkaline phosphatase conjugated anti-rabbit IgG and Nitro blue tetrazolium (Figures 3A and 3B). See Example 10 for more detail on the antisera preparation. The peptide antisera detect a single protein from *E. coli* (Fig. 3A) and a heterogeneous set of proteins ranging in size from 24-32 kDa from COS cells (Fig. 3B). Binding of radiolabeled TGF-β to proteins expressed by transfected COS-M6 cells was performed essentially as described previously (Segarini et al. (1989) Mol. Endocrinol. 3:261-272).

Briefly, purified TGF-β was radiolabeled with $Na^{125}I$ and incubated with aliquots of the conditioned media from cells transfected with either plasmid pSG/βRIIs or pSG5 as a negative control. Following affinity labeling, TGF-β/soluble receptor complexes were covalently crosslinked with disuccinimidyl suberate, separated by reducing SDS-PAGE and visualized by autoradiography. Included in some of the binding reactions was a 1000-fold molar excess of unlabeled TGF-β to compete for binding with radiolabeled material. Fig. 2B is the result of such a binding experiment demonstrating the presence of an affinity labeled protein of approximately 40 kda that was not present in vector-only transfected cells. Binding of radiolabeled TGF-β1 could be effectively blocked by including a 1000-fold molar excess of unlabeled TGF-β1 (Fig. 2B), lane labeled pSG/βRIIs$^+$) but not TGF-β2 (data not shown) in the binding reaction mixture.

Example 7

The effect of sβ-RII is compared with that of anti-TGF antibody in a glomerulonephritis model. Experimental glomerulonephritis can be induced in rats with a single injection of antithymocyte serum because the glomerular mesangial cells express a thy-1.1 epitope on their surfaces. The experimental lesion is acute mesangial proliferative glomerulonephritis and is characterized by expansion of the mesangial matrix and hypercellularity. The injured cells also express more TGF-β1 mRNA and TGF-β1, which in turn stimulates the synthesis of two proteoglycans, biglycan and decorin.

The antiserum is prepared by immunizing a rabbit with a cyclized, synthetic peptide containing residues 78-109 of human TGF-β1. The anti-TGF-β1 antiserum is capable of inhibiting binding of TGF-β to cells. (Flanders et al. (1988) Biochemistry 27:739-46)

First, glomerulonephritis is induced in rats by an intravenous injection of antithymocyte serum. Next, for six days, three groups of rats are treated with daily intravenous injections of saline (the negative control group), anti-TGF-β1 antiserum (the positive control group) or sβ-RII.

On the seventh day, the animals are sacrificed and slides are made of the kidneys, which are stained with periodic acid-Schiff solution to emphasize the pathological changes. The negative control kidneys have full-blown glomerulonephritis with reddish-pink amorphous fibrous material filling most of the glomerulus. The positive control kidneys have a staining pattern which is similar to a normal glomerulus. The kidney which is treated with sβ-RII also has a normal appearance, indicating that the sβ-RII blocks the response due to the secretion of excessive TGF-β.

The extent of glomerular injury can be quantitated by performing glomerular cell counts from 30 randomly selected glomeruli from normal animals and nephritic animals in each group. On day 4, there are fewer cells in glomeruli from antithymocyte-treated rats, presumably because the treatment causes cell lysis. By day 7, there are more cells than normal. The changes in cell counts in the anti-TGF-β1 and sβ-RII group are expected to be the same.

Another measure of the effect of anti-TGF-β1 and sβ-RII on the disease process is to quantitate the amount of extracellular matrix accumulation in the glomeruli. The degree of glomerular matrix expansion is determined as the percentage of each glomerulus occupied by the mesangial matrix according to the method of Raij et al. (1984) Kidney Int. 26: 137-43. The anti-TGF-β1 and sβ-RII kidneys are expected to have similar percentages of mesangial matrix to that in normal kidney, and significantly less mesangial matrix than in the negative control kidneys.

After glomerular injury and simultaneous treatment with anti-TGF-β, the mesangial cells expressed more TGF-β1 mRNA; however, proteoglycan synthesis is nearly normal with anti-TGF-β1 and sβ-RII.

Example 8

The following compares the action of TGF-β antibody with sβ-RII in an arthritis model. TGF-β antibody is prepared as disclosed in U.S. Application Serial No. 759,109, which is incorporated by reference in its entirety. This application discloses the formation of monoclonal antibodies 3C7.14 specific for TGF-β2 and TGF-β3 and 1D11.16 specific for TGF-β1, -β2 and -β3.

First, arthritis is induced in pathogen-free female LEW rats (Harlan Sprague Dawley, Indianapolis, IN)

weighing about 100 grams. Each receives a dose of cell wall fragments from Group A streptococci (SCW) (30 μg rhamnose/gm body weight), injected intraperitoneally (ip) according to the technique described in Brandes et al. (1991) J. Clin. Invest. 87:1108.

SCW-injected and control LEW rats are given an intraarticular (IA) injection in one of the hind ankles of one of the following:

1. anti-TGF-β (1D11.16) which is specific for TGF-β1 and TGF-β1 in 25 μl PBS,
2. sβ-RII in 25 μl PBS,
3. PBS only, or
4. an irrelevant isotype control mouse myeloma immunoglobulin (MOPC21, IgG_1)

Joints are clinically monitored by determining the amount of joint erythema, swelling and distortion on a scale of 0 (normal) to 4 (severe inflammation). Radiographs are taken and are evaluated for soft tissue swelling, joint space narrowing, bone erosions and deformity. Tissue specimens are obtained and prepared for histopathologic analysis as described in Brandes et al., ibid. Total RNA is isolated from excised synovial tissues according to the method of Allen et al. (1990) J. Exp. Med. 171:231.

Injection of SCW produces an acute inflammatory response which is clinically detectable within hours and maximal in 3-5 days. When anti-TGF-β is injected directly into a joint before ip administration of the SCW, inflammation at 24 hours is significantly below that observed in joints with the irrelevant antibody. At the peak of the acute response, inflammation of anti-TGF-β joints remains far below that of joints with the irrelevant antibody. Even if joints are injected with anti-TGF-β when inflammation is well developed (day 13), anti-TGF-β still has a significant anti-inflammatory

effect, when compared to irrelevant antibody. Because sβ-RII also binds TGF-β, sβ-RII has a similarly beneficial effect when given early or late in the inflammatory process.

Example 9

To simulate acute liver injury, the hepatotoxin, D-galactosamine, is administered to cause liver fibrosis, mortality, and maximal TGF-β gene expression approximately 48 hours after administration. A rat model utilizing this hepatotoxin is used to evaluate the therapeutic effect of sβ-RII on acute liver fibrosis and serves as a model for liver cirrhosis.

Sprague-Dawley rats are administered 1.6 g/kg D-galactosamine intraperitoneally. Half of the rats are also to be given sβ-RII two hours prior to D-galactosamine administration, and at 24, 48, and 72 hours after D-galactosamine administration. Two rats from each test group are sacrificed at 48 hours to evaluate the efficacy of sβ-RII at peak TGF-β gene expression.

Histological examination reveals that sβ-RII-treated animals exhibit reduced liver pathology. Northern blot evaluation of tissues from specimens treated with sβ-RII show significantly decreased levels of collagen mRNA and almost normal levels of serum albumin, in contrast to non-treated controls.

Example 10

Polyclonal antibodies to the carboxy-terminal region of sβ-RII have been prepared and tested. Linear or cyclic peptides of amino acids 68-89 were injected once per month (in one case a month was skipped) at a concentration of 4 mg/ml in phosphate buffered saline with 200 μl per injection in complete Freund's adjuvant

(first injection) or incomplete Freund's adjuvant (succeeding boosts). Bleeds were collected during the second, fourth, fifth, sixth, and seventh months after the initial boost.

Soluble and inclusion body fractions from *E. coli*, and supernatant fractions from sβ-RII and vector-transfected COS cells were electrophoresed under reducing conditions on SDS-PAGE. The *E. coli* fractions were prepared in duplicate. The proteins were electrotransferred to Immobilon P filters (Millipore, Bedford MA). Next, the *E. coli* filters were blocked with super-Blotto (2.5% nonfat dry milk in Tris buffered saline (TBS), 10% (v,v) glycerol, 1M glucose, 0.5% Tween 20). The COS filters were blocked with standard Blotto (5% nonfat dry milk in Tris buffered saline). Then all filters were reacted with 10 μg/ml of rabbit antiserum. One half of the duplicate *E. coli* filters was incubated with nonimmune rabbit antiserum as a control. The blots were washed with TBS, containing 0.05% Tween 20 (T-TBS), and reacted with a secondary antibody, goat anti-rabbit IgG horseradish peroxidase (HRP) diluted 1:50,000, and incubated for one hour. The blot was washed with T-TBS and reacted with a membrane TMB kit for Kirkegaard and Perry according to package directions.

Figure 3A shows *E. coli* soluble and inclusion body fractions reacted with peptide antiserum (left lane) or with control rabbit antisera (right lane). Figure 3B shows COS supernatants from cells transfected with control vector (right lane) or sβ-RII vector (left lane). sβ-RII produced in COS cells is glycosylated and appears as heterogeneous bands.

In both *E. coli* and COS systems, a soluble receptor was produced and included an amino acid sequence that is recognized by antisera induced by linear and/or cyclic peptides of amino acids 68-89 of the extracellular

domain of the type II TGF-β receptor. Control nonimmune rabbit antiserum did not recognize the type II receptor. Vector-transfected COS cells did not produce a protein that is reactive with the antisera.

COS cells were transfected with the pSG/βRII vector or with a control vector (pSG5). After 72 hours, the two supernatants were collected, incubated with 50 pM ^{125}I-TGF-β1 without (-) or with (+) 40 nM unlabeled TGF-β1. The samples were crosslinked with 0.3mM disuccinimidyl suberate (DSS) (Pierce Chemical Co., Rockford IL) and electrophoresed on SDS-PAGE under reducing conditions. The gels were dried and exposed for autoradiography, with the result shown in Figure 2B. A single asterisk (*) indicates monomeric TGF-β1, a double asterisk indicates dimeric TGF-β1 and a single arrowhead indicates the TGF-β1/sβ-RII complex. The size and heterogeneous appearance of the complex suggests that the sβ-RII from COS cells is glycosylated with 7-10 kD of carbohydrate.

Example 11

The efficacy of TGF-ß in preventing post-radiation fibrosis can be assessed in patients who require radiation therapy for a tumor, such as adenocarcinoma, prior to bowel resection. At surgery, biopsies of various tissues can be obtained. There are two negative control groups: patients who do not receive radiotherapy prior to surgery and patients who receive radiotherapy but no TGF-ß. The study group is administered sß-RII concomitantly with the radiotherapy.

Each day, when the patient reports for radiotherapy, the patient receives an intravenous injection of sß-RII. After radiation therapy is stopped, the patient receives weekly intravenous doses of sß-RII until surgery.

At surgery, the tumor and associated tissues are removed. Slides are made from the tumor and tissue samples. Under microscopic examination, the tissue samples show signs of healing without excessive fibrosis.

This invention has been detailed both by example and by direct description. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above. Those equivalents are to be included within the scope of this invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SEGARINI, PATRICIA R.
DASCH, JAMES R.
OLSEN, DAVID R.
CARRILLO, PEDRO A.

(ii) TITLE OF INVENTION: USES OF TGF-BETA RECEPTOR FRAGMENT AS A THERAPEUTIC AGENT

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Morrison & Foerster
(B) STREET: 755 Page Mill Road
(C) CITY: Palo Alto
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94304-1018

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US UNASSIGNED
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: LUTHER, BARBARA J.
(B) REGISTRATION NUMBER: 33,954
(C) REFERENCE/DOCKET NUMBER: 22095-20261.20

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 813-5600
(B) TELEFAX: (415) 494-0792

(C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2095 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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60

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120

CTCCTGTGCA GCTTCCCTCG GCCGCCGGGG GCCTCCCCGC GCCTCGCCGG CCTCCAGGCC
180

CCTCCTGGCT GGCGAGCGGG CGCCACATCT GGCCCGCACA TCTGCGCTGC CGGCCCGGCG
240

CGGGGTCCGG AGAGGGCGCG GCGCGGAGCG CAGCCAGGGG TCCGGGAAGG CGCCGTCCGT
300

GCGCTGGGGG CTCGGTCTAT GACGAGCAGC GGGGTCTGCC ATGGGTCGGG GGCTGCTCAG
360

GGGCCTGTGG CCGCTGCACA TCGTCCTGTG GACGCGTATC GCCAGCACGA TCCCACCGCA
420

CGTTCAGAAG TCGGTTAATA ACGACATGAT AGTCACTGAC AACAACGGTG CAGTCAAGTT
480

TCCACAACTG TGTAAATTTT GTGATGTGAG ATTTTCCACC TGTGACAACC AGAAATCCTG
540

CATGAGCAAC TGCAGCATCA CCTCCATCTG TGAGAAGCCA CAGGAAGTCT GTGTGGCTGT
600

ATGGAGAAAG AATGACGAGA ACATAACACT AGAGACAGTT TGCCATGACC CCAAGCTCCC
660

CTACCATGAC TTTATTCTGG AAGATGCTGC TTCTCCAAAG TGCATTATGA AGGAAAAAAA
720

AAAGCCTGGT GAGACTTTCT TCATGTGTTC CTGTAGCTCT GATGAGTGCA ATGACAACAT
780

CATCTTCTCA GAAGAATATA ACACCAGCAA TCCTGACTTG TTGCTAGTCA TATTTCAAGT
840

GACAGGCATC AGCCTCCTGC CACCACTGGG AGTTGCCATA TCTGTCATCA TCATCTTCTA
900

CTGCTACCGC GTTAACCGGC AGCAGAAGCT GAGTTCAACC TGGGAAACCG GCAAGACGCG
960

GAAGCTCATG GAGTTCAGCG AGCACTGTGC CATCATCCTG GAAGATGACC GCTCTGACAT
1020

CAGCTCCACG TGTGCCAACA ACATCAACCA CAACACAGAG CTGCTGCCCA TTGAGCTGGA
1080

CACCCTGGTG GGGAAAGGTC GCTTTGCTGA GGTCTATAAG GCCAAGCTGA AGCAGAACAC
1140

TTCAGAGCAG TTTGAGACAG TGGCAGTCAA GATCTTTCCC TATGAGGAGT ATGCCTCTTG
1200

GAAGACAGAG AAGGACATCT TCTCAGACAT CAATCTGAAG CATGAGAACA TACTCCAGTT
1260

CCTGACGGCT GAGGAGCGGA AGACGGAGTT GGGGAAACAA TACTGGCTGA TCACCGCCTT
1320

CCACGCCAAG GGCAACCTAC AGGAGTACCT GACGCGGCAT GTCATCAGCT GGGAGGACCT
1380

GCGCAAGCTG GGCAGCTCCC TCGCCCGGGG GATTGCTCAC CTCCACAGTG ATCACACTCC
1440

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1500

GAAGAACGAC CTAACCTGCT GCCTGTGTGA CTTTGGGCTT TCCCTGCGTC TGGACCCTAC
1560

TCTGTCTGTG GATGACCTGG CTAACAGTGG GCAGGTGGGA ACTGCAAGAT ACATGGCTCC
1620

AGAAGTCCTA GAATCCAGGA TGAATTTGGA GAATGCTGAG TCCTTCAAGC AGACCGATGT
1680

CTACTCCATG GCTCTGGTGC TCTGGGAAAT GACATCTCGC TGTAATGCAG TGGGAGAAGT
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1800

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1860

CCAGGGCATC CAGATGGTGT GTGAGACGTT GACTGAGTGC TGGGACCACG ACCCAGAGGC
1920

CCGTCTCACA GCCCAGTGTG TGGCAGAACG CTTCAGTGAG CTGGAGCATC TGGACAGGCT
1980

CTCGGGGAGG AGCTGCTCGG AGGAGAAGAT TCCTGAAGAC GGCTCCCTAA ACACTACCAA
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2095

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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49

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGATCCGA TAGTGGAGGA TGATTAAATG ATCCCACCGC ACGTTCAGAA GT
52

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGAATTCA AGCTTAGTCA GGATTGCTGG TGTTATATTC TTCTGA
46

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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33

CLAIMS

1. A method for treating an individual for a medical condition associated with TGF-β excess comprising administering parenterally, orally or locally a sufficient amount of TGF-β-binding receptor fragment to the individual to reduce excess TGF-β activity in the individual.

2. The method of claim 1 wherein the TGF-β receptor fragment comprises a fragment of recombinant human TGF-β receptor.

3. The method of claim 1 wherein the TGF-β receptor fragment is administered by a method selected from the group consisting of intravenous, intraocular, intraarticular, transdermal, and enteral administration.

4. The method of claim 1 wherein the TGF-β receptor is type II.

5. The method of claim 1 wherein the TGF-β receptor is type I.

6. The method of claim 1 wherein the TGF-β receptor is type III.

7. The method of claim 1 wherein said medical condition comprises cancer.

8. The method of claim 7 wherein said cancer is plasmacytoma, glioblastoma, astrocytoma, or prostatic or ovarian carcinoma.

9. The method of claim 1 wherein said medical condition comprises a collagen vascular disease.

10. The method of claim 9 wherein said collagen vascular disease is systemic sclerosis, polymyositis, scleroderma, or dermatomyositis.

11. The method of claim 1 wherein said medical condition comprises an autoimmune disease.

12. The method of claim 11 wherein said autoimmune disease is rheumatoid arthritis or systemic lupes erythematosus.

13. The method of claim 1 wherein said medical condition comprises a fibroproliferative disorder.

14. The method of claim 13 wherein said fibroproliferative disorders comprise hepatic, kidney, intraocular and pulmonary fibrosis.

15. The method of claim 13 wherein said fibroproliferative disorder is selected from the group consisting of diabetic nephropathy, glomerulonephritis, proliferative vitreoretinopathy, liver cirrhosis, biliary fibrosis, and myelofibrosis.

16. The method of claim 1 wherein the condition of TGF-β excess is characterized by immunosuppression associated with an infectious disease.

17. The method of claim 16 wherein the immunosuppression occurs with a trypanosomal infection.

18. The method of claim 16 wherein the immunosuppression occurs with a viral infection selected from the group consisting of human immunosuppression virus, HTLV-1, lymphocytic choriomeningitis virus, and hepatitis.

19. A method of increasing the effectiveness of a vaccine comprising administering to an individual about to receive a vaccine or receiving a vaccine a sufficient amount of TGF-β-binding receptor fragment to increase the immune response to the vaccine in the individual.

20. The method of claim 19 wherein the individual is an immunocompromised individual.

21. A method of treating a wound in an individual to avoid excessive connective tissue formation associated with TGF-β excess, the method comprising administering a sufficient amount of TGF-β-binding receptor fragment to the individual to reduce the excess of TGF-β in the individual.

22. The method of claim 21 wherein the wound is a surgical incision or trauma-induced laceration.

23. The method of claim 21 wherein the wound involves the peritoneum and the excessive connective tissue formation comprises abdominal adhesions.

24. The method of claim 21 wherein the excessive connective tissue is selected from the group consisting of a scar, hypertrophic scar, and a keloid.

25. The method of claim 24 wherein the scar involves restenosis of blood vessels.

26. A method of preventing postradiation fibrosis in an individual undergoing or about to undergo radiation therapy, the method comprising administering to the individual TGF-β-binding receptor fragment in amounts sufficient to prevent excessive fibrous tissue formation.

AMENDED CLAIMS
[received by the International Bureau
on 08 April 1994 (08.04.94);
original claims 1, 19, 21 and 26 amended;
remaining claims unchanged (3 pages)]

1. A method for treating an individual for a medical condition associated with TGF-β excess comprising administering parenterally, orally or locally a sufficient amount of a high affinity TGF-β-binding receptor fragment to the individual to reduce excess TGF-β activity in the individual.

2. The method of claim 1 wherein the TGF-β receptor fragment comprises a fragment of recombinant human TGF-β receptor.

3. The method of claim 1 wherein the TGF-β receptor fragment is administered by a method selected from the group consisting of intravenous, intraocular, intraarticular, transdermal, and enteral administration.

4. The method of claim 1 wherein the TGF-β receptor is type II.

5. The method of claim 1 wherein the TGF-β receptor is type I.

6. The method of claim 1 wherein the TGF-β receptor is type III.

7. The method of claim 1 wherein said medical condition comprises cancer.

8. The method of claim 7 wherein said cancer is plasmacytoma, glioblastoma, astrocytoma, or prostatic or ovarian carcinoma.

18. The method of claim 16 wherein the immunosuppression occurs with a viral infection selected from the group consisting of human immunosuppression virus, HTLV-1, lymphocytic choriomeningitis virus, and hepatitis.

19. A method of increasing the effectiveness of a vaccine comprising administering to an individual about to receive a vaccine or receiving a vaccine a sufficient amount of a high affinity TGF-β-binding receptor fragment to increase the immune response to the vaccine in the individual.

20. The method of claim 19 wherein the individual is an immunocompromised individual.

21. A method of treating a wound in an individual to avoid excessive connective tissue formation associated with TGF-β excess, the method comprising administering a sufficient amount of a high affinity TGF-β-binding receptor fragment to the individual to reduce the excess of TGF-β in the individual.

22. The method of claim 21 wherein the wound is a surgical incision or trauma-induced laceration.

23. The method of claim 21 wherein the wound involves the peritoneum and the excessive connective tissue formation comprises abdominal adhesions.

24. The method of claim 21 wherein the excessive connective tissue is selected from the group consisting of a scar, hypertrophic scar, and a keloid.

25. The method of claim 24 wherein the scar involves restenosis of blood vessels.

26. A method of preventing postradiation fibrosis in an individual undergoing or about to undergo radiation therapy, the method comprising administering to the individual a high affinity TGF-β-binding receptor fragment in amounts sufficient to prevent excessive fibrous tissue formation.

STATEMENT UNDER ARTICLE 19

Applicant respectfully submits that the above amendment to claim 1 more clearly defines the invention. Support for the added phrase is found on page 10, lines 23-31.

The claimed inventions involve an inventive step because no one reference cited in the International Search Report nor any combination of these references teach or suggest applicant's inventive method. More particularly, applicant's invention is directed to a method for treating an individual with a medical condition associated with excess TGF-β. Applicant's method comprises the administration of a high affinity TGF-β binding receptor fragment in order to reduce the excess TGF-β found in the individual having the medical condition. The cited prior art does not adequately teach or suggest this therapeutic use of a high affinity TGF-β receptor fragment.

More specifically, the Border et al. (1992) reference at best can only be viewed as an invitation to experiment with soluble forms of TGF-β receptors. As stated on page 6, first column, first paragraph of the Border reference, "soluble forms of the receptors may also inhibit TGF-β activity by the same mechanism <u>but this has not yet been proven</u>. (emphasis added) The Smith et al. (1987) reference teaches only the use of soluble CD_4 for binding to the HIV-1 envelope glycoprotein gp120. These results can not be extrapolated to the therapeutic use of TGF-β-receptor fragments. The Andres et al. (1989) and Lin et al. (1992) references do not teach the therapeutic use of TGF-β receptor fragments. While the remainder of the cited references discuss the role of TGF-β in various medical conditions, applicant can not find a teaching or suggestion directed to the therapeutic use of a high affinity TGF-β-binding receptor fragment in these references.

In view of the above, applicant respectfully submits that the claimed inventions contain an inventive step over the prior art.

FIG. 1

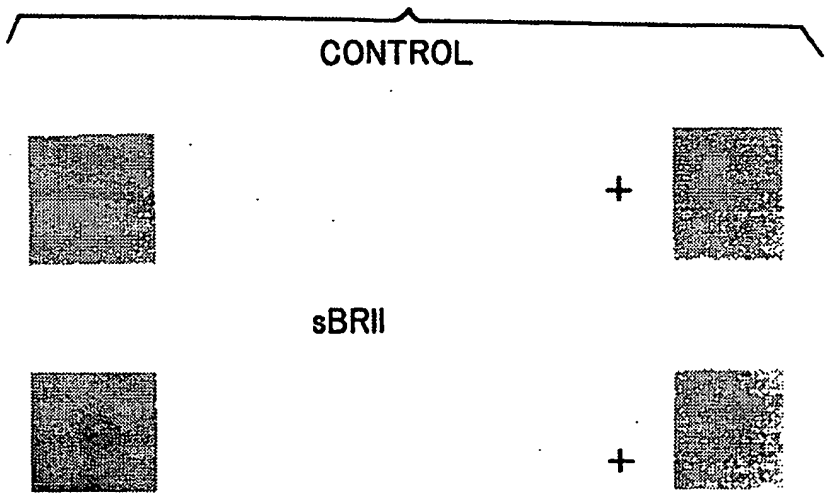
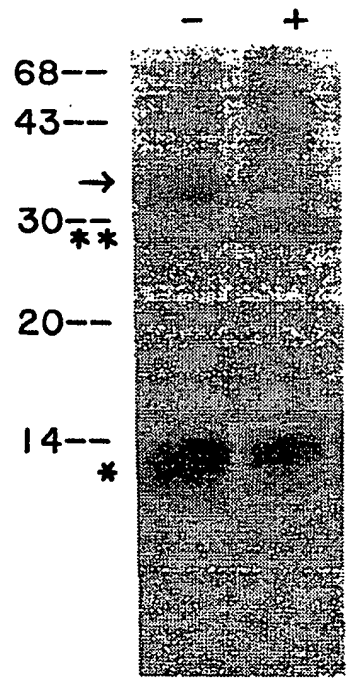


FIG. 2A



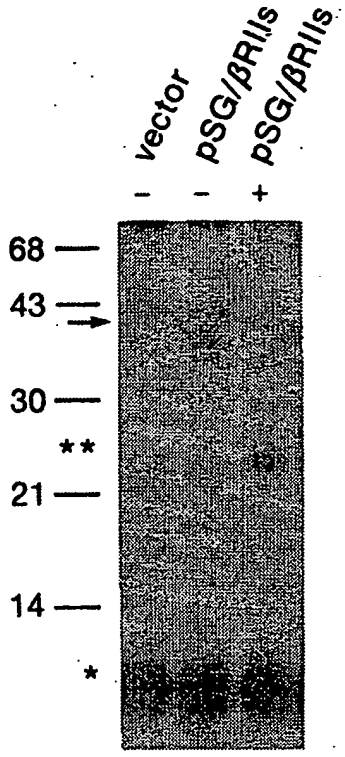


FIG. 2B

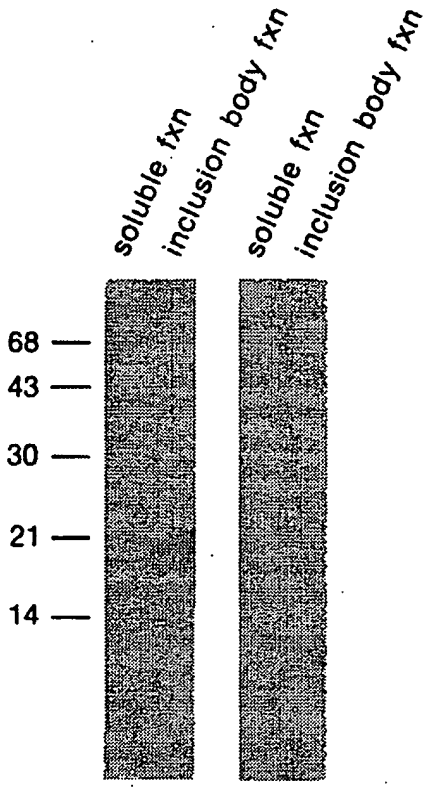


FIG. 3A

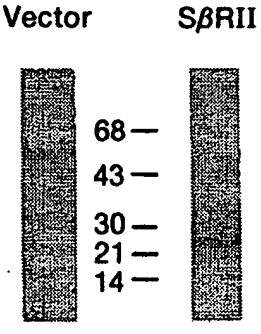


FIG. 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10455

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/43, 37/02
US CL :514/2, 8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Cell Biology, Volume 109, Number 6, Part 1, issued December 1989, J. L. Andres et al., "Membrane-anchored and Soluble Forms of Betaglycan, a Polymorphic Proteoglycan that Binds Transforming Growth Factor-beta", pages 3137-45, especially the abstract.	1-24
Y	Journal of Clinical Investigation, Volume 90, issued July 1992, W. A. Border et al., "Transforming Growth Factor-beta in Disease: The Dark Side of Tissue Repair", pages 1-7, especially page 1, col. 2, and page 5, col.2 to page 6, col. 1.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be part of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 26 January 1994	Date of mailing of the international search report 08 FEB 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10455

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 238, issued 18 December 1987, D. H. Smith et al., "Blocking of HIV-1 Infectivity by a Soluble, Secreted Form of the CD4 Antigen", pages 1704-07, especially the abstract; page 1704, col. 2; and page 1705, col. 3 to page 1706, col. 1.	1-24
Y	Cell, Volume 68, issued 21 February 1992, H. Y. Lin et al., "Expression Cloning of the TGF-beta Type II Receptor, a Functional Transmembrane Serine/Threonine Kinase", pages 775-85, especially the abstract and Fig. 2.	1-4, 7-24
Y	Cell, Volume 67, issued 15 November 1991, F. Lopez-Casillas et al., "Structure and Expression of the Membrane Proteoglycan Betaglycan, a Component of the TGF-beta Receptor System", pages 785-95, especially pages 791-92.	1-3, 6-24
Y	Cancer Investigation, Volume 9, Number 3, issued 1991, M. A. Liu et al., "Transforming Growth Factor-beta--Mullerian Inhibiting Substance Family of Growth Regulators", pages 325-36, especially Table I.	1-24
Y	Lancet, Volume 336, issued 15 December 1990, G. F. Whalen, "Solid tumours and wounds: transformed cells misunderstood as injured tissue?", pages 1489-92, see the entire document.	1-8
Y	Immunology Today, Volume 10, Number 8, issued 1989, S. M. Wahl et al., "Inflammatory and immunomodulatory roles of TGF-beta", pages 258-61, see the entire document.	1-6, 11, 12, 16-20
Y	Annals of the New York Academy of Sciences, Volume 593, issued 1990, R. L. Wilder et al., "Transforming growth factor-beta in rheumatoid arthritis", pages 197-207, especially the abstract.	1-6, 11, 12
Y	Journal of Cell Biology, Volume 108, issued June 1989, M. J. Czaja et al., "In Vitro and In Vivo Association of Transforming Growth Factor-beta-1 with Hepatic Fibrosis", pages 2477-82, especially the abstract.	1-6, 13, 14, 16
Y	Nature, Volume 346, issued 26 July 1990, W. A. Border et al., "Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta-1", pages 371-74, especially the abstract.	1-6, 13-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10455

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	American Journal of Respiratory Cell and Molecular Biology, Volume 5, issued 1991, N. Khalil et al., "Increased Production and Immunohistochemical Localization of Transforming Growth Factor-beta in Idiopathic Pulmonary Fibrosis", pages 155-62, especially the abstract.	1-6, 13, 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10455

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DATABASES: USPTO-APS, Medline, Biosis, SciSearch, Derwent WPI
SEARCH TERMS: TGF- or Transforming Growth Factor-Beta; cancer or neoplasm; collagen; autoimmune; immunosuppress? or immunodeficiency; vaccine or adjuvant; fibrosis or glomerulonephritis; wound heal? or scar?